Studies of Glucocorticoid Resistance in Chronic Lymphocytic Leukaemia

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by

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Contents

Abstract

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Glucocorticoids represent an important component of modern treatment regimens for patients with fludarabine-refractory or *TP53*-defective chronic lymphocytic leukaemia. However, not all patients respond to GC therapy. This *in-vitro* study investigated the molecular mechanisms responsible for GC resistance in a cohort of 46 primary CLL samples. As expected, dexamethasone-induced killing was more pronounced in cases with UM-*IGHV* genes. Cross resistance was observed between dexamethasone and other GCs but not fludarabine, implying distinct resistance mechanisms. GC resistance was not associated with GC receptor defects, altered NF-κB signalling or impaired Bim up-regulation. siRNA knockdown experiments confirmed that Bim plays a crucial role in the GC-induced killing of CLL cells, while immunoprecipation experiments showed that GC resistance was associated with impaired Bax/Bak activation and that Bim was bound to Bcl-2. Levels of Bcl-2 were found to be higher in GC-resistant samples, and disruption of the Bim/Bcl-2 interaction by ABT-737 sensitised GC-resistant CLL cells to GC-induced killing. Taken together, these findings provide compelling evidence that GC resistance in CLL cells results from the sequestration of up-regulated Bim by high levels of Bcl-2 with consequent failure of Bax/Bak activation and apoptosis induction.

List of figures

List of tables

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Declaration

The baseline GR and Bax/Bak protein expression screening was performed by Miss N Rockliffe. The Bim knockdown, and elements of the Bcl-2 family immunoprecipitation work was performed by Dr Z Zhuang. The TRAC assay drug sensitivity screening was performed in collaboration with Dr AG Bosanquet and biomedical scientists at Bath Royal United Hospital Cancer Research. Otherwise, the work presented in this thesis is my own. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification.

List of abbreviations

AIF	Apoptosis-inducing factor
ALL	Acute lymphoblastic leukaemia
AP-1	Activator protein-1
ATM	Ataxia telangiectasia-mutated gene
B cell	B lymphocyte
BCR	B cell receptor
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complimentary deoxyribonucleic acid
CDK	Cyclin dependent kinase
CLL	Chronic lymphocytic leukaemia
CO_2	Carbon dioxide
CpG-ODN	Deoxyribo(cytidine-phosphate-guanosine) motif containing oligodeoxynucleotide
CR	Complete remission
CTD	C-terminal domain
Dex	Dexamethasone
$DIOC_6$	3,3-dihexyloxacarbocyanine iodide
DLC	Dynein light chain
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleoside triphosphates
ECL	Electrochemiluminescence
ELISA	Enzyme Linked Immunosorbant Assay
EMSA	Electrophoretic Mobility Shift Assay
EtBr	Ethidium bromide
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FISH	Fluorescent in-situ hybridisation
FITC	Fluorescein isothiocyanate
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GSK-3	Glycogen synthase kinase 3
h	Hour(s)
Hb	Haemoglobin
HDAC	Histone deacetylase
HDMP	High-dose glucocorticoids
HRP	Horse-radish peroxidase
HSCT	Haematological stem-cell transplant
IκB	Inhibitor of κB
IFN	Interferon
Ig	Immunoglobulin
IGHV	Variable region of immunoglobulin heavy chain gene
IKK	IκB kinase

IL	Interleukin
IP	Immunoprecipitation
kDa	Kilo-Dalton
LBD	Ligand-binding domain
LC	Lymphocyte count
M	Mutated
mAb	Monoclonal antibody
mRNA	Messenger RNA
MAPK	Mitogen-activated protein kinase
Mep	6-methylprednisolone
MFI	Mean fluorescence intensity
min	Minute(s)
miR	MicroRNA
MM	Multiple myeloma
mRNA	Messenger mRNA
N/A	Non-applicable
nGRE	Negative glucocorticoid response element
NF-κB	Nuclear factor κB
NFAT	Nuclear factor of activated T cells
NLS	Nuclear localisation sequence
ORR	Overall response rate
OS	Overall survival
TP53	p53 gene (human)
pAb	Polyclonal antibody
PAGE	poly-acrylamide gel electrophoresis
PARP	poly-ADP ribose polymerase
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFS	Progression-free survival
PI	Propidium iodide
PI3-K	Phophatidly inositol phosphatase 3
PKC	Protein kinase C
Poly-HEMA	Poly(2-hydroxyehtyl methacrylate)
PR	Partial remission
PS	Phosphatidylserine
PVDF	Polyvinylidine fluoride
qRT-PCR	Quantitative Real-Time PCR
RCF	Relative centrifugal force
RIC	Reduced intensity conditioning
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
s	Second(s)
SD	Standard deviation
SDF-1	Stromal cell derived factor 1
SDS	Sodium dodecadyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
STAT	Signal transducer and activator of transcription

t0	Time zero
TBE	Tris/borate/EDTA
TBS	Tris-buffered saline
TBS-T	TBS-Tween
TFI	Treatment free interval
TLR	Toll-like receptor
TNF	Tumour necrosis factor
UM	Unmutated
WBC	White blood cells
WCL	Whole cell lysate
wt	Wild type
Zap-70	Zeta-chain-associated protein kinase 70

Chapter 1: General Introduction

1.1 Chronic lymphocytic leukaemia

1.1.1 Definition

CLL is defined by the accumulation of circulating clonal CD23+ CD5+ light-chain-restricted B cells in the blood to a concentration of $\geq 5x10^9/L$ (Gribben 2010), persisting for over 3 months. CLL lymphocytes also commonly express CD19 and show reduced levels of IgM, IgD, and CD79b (Moreau, Matutes et al. 1997). It is diagnosed by an absolute increase in lymphocytosis and/or bone marrow infiltration, coupled with the characteristic features of morphology and immunophenotype. Patients may present with lymphadenopathy, systemic symptoms such as tiredness, night sweats and weight loss, or the symptoms of anaemia or infection. 70-80% of patients are diagnosed as an incidental finding on a routine full blood count.

1.1.2 Epidemiology

CLL has an annual incidence of over 3 per 100,000 (Dores, Anderson et al. 2007), rising to 50 per 100,000 per year over the age of 70. CLL is the most prevalent cancer of the haematopoietic system in the western world, with high associated mortality. In fact, 70% of CLL patients die from causes related to CLL, especially in the elderly. Many fatalities in CLL are caused by infection and haemorrhage due to pancytopenia or immunosuppressive treatments (Anaissie, Kontoyiannis et al. 1998), and secondary cancers. Immunological aberrations complicate the management of CLL (Mauro, Foa et al. 2000). Median age at

diagnosis is approximately 72 years (Dores, Anderson et al. 2007) and median survival is 5-10 years. The disease is roughly twice as common in males than females (Gribben 2010), and there is evidence of familial clustering in a minority of cases (Houlston, Catovsky 2008).

1.1.3 Prognosis and prognostic factors

The clinical course of CLL is highly variable, with some patients remaining asymptomatic for many years, whilst others show aggressive progression (Gribben 2010). The disease progresses from an indolent lymphocytosis to one of generalised lymphatic enlargement with pancytopenia (Anaissie, Kontoyiannis et al. 1998). Dysfunctional haematopoiesis leads to accumulation of immunologically inactive lymphocytes in blood, bone marrow, and lymphatic tissues, which can cause infection, anaemia, and thrombocytopenia (Zenz, Mertens et al. 2010). The disease is currently incurable by conventional treatments and so many patients experience a range of treatments during the course of the disease.

Due to the toxicity of CLL treatments, predicting good prognosis is of essential importance in CLL management. There has been great interest in recent years in identifying ways to predict clinical outcome. Initially, clinical staging systems were developed to assess the degree of tissue infiltration and marrow suppression. The two most widely used systems in CLL are those developed by Rai (Rai, Sawitsky et al. 1975) and Binet (Binet, Auquier et al. 1981) (Tables 1.1 A and 1.1 B), which depend upon measurements of total tumour mass as determined by measurements of lymphadenopathy, organomegaly, platelet, and haemoglobulin

values (Hamblin 2007). These staging systems are still used to predict prognosis as well as to assess disease progression, and to guide decisions over when to begin therapy, but they are insensitive to clinical heterogeneity within early CLL and so are now supplemented by a range of laboratory prognostic markers. Adding parameters such as age, gender, lymphocyte count and serum markers can also provide a better prediction of overall survival (Wierda, O'Brien et al. 2007, Shanafelt, Jenkins et al. 2009) and time to first treatment in early stage CLL (Molica, Mauro et al. 2010, Bulian, Tarnani et al. 2010). Lymphocyte doubling time is also still an important prognostic factor in CLL, as patients with birth rates >0.35% of the clonal population per day have more active and progressive disease (Molica, Alberti 1987, Messmer, Messmer et al. 2005).

Table 1.1 A and 1.1 B. The Rai and Binet staging systems showing median survival as described in the original papers. This demonstrates the marked effect that tissue infiltration has on prognosis.

Table 1.1 A – Rai Staging

Stage	Clinical Features	Average Survival (months)
0	Bone marrow and blood lymphocytosis only	>150
I	Lymphocytosis with enlarged nodes	101
II	Lymphocytosis with enlarged spleen or liver or both	71
III	Lymphocytosis with anaemia (Hb less than 10g)	19
IV	Lymphocytosis with thrombocytopenia (platelets less than 100,000/mm^3)	19

Table 1.1 B– Binet Staging

Stage	Clinical Features	Average Survival (months)
A	No anaemia, no thrombocytopenia, less than three involved areas (e.g. spleen, liver, lymph nodes)	Same as controls
B	No anaemia, no thrombocytopenia, three or more involved areas	84
C	Anaemia and/or thrombocytopenia	24

Identification of poor prognosis is a key area of research in CLL. Overall, (unmutated) UM-*IGHV* genes, use of the *IGHV-3-21* gene, deletion of 11q and a raised serum B2 microglobulin correlate with reduced PFS (progression-free survival) and OS (overall survival) in clinical trials of alkylating agent and purine analogue treatment. *IGHV* mutation status, and CD38 and Zap-70 expression are routinely assessed to aid in identifying poor prognosis patients (Hamblin 2007). *IGHV* status segregates the disease into more benign and more malignant versions (Hamblin 2007), and is by far the most useful indicator of disease progression for

newly diagnosed patients. To determine *IGHV* status CLL cells are studied by gene sequencing. Comparison of the sequence obtained with that of germline is then performed and the percentage deviation from the germline sequence calculated. Clones are described as either mutated (M) or unmutated (UM) depending on whether they exhibit ≥2% or <2% deviation from germline. These sub-groups have markedly different median survivals of around 24 and 8 years, respectively (Hamblin, Davis et al. 1999). A search for surrogate markers for this difficult assay led to a flow cytometric assay for CD38 (Hamblin 2007). CD38 is a hydrolase ectoenzyme expressed on the surface of B, T, and NK cells, which plays a role in adhesion and signal transduction (Matrai 2005). The original cut-off for CD38 positivity was 30%, and this is still upheld, though it is not uncontested (Matrai 2005). A flow cytometric assay is also available to measure ZAP-70 expresssion, as poor prognosis also correlates with the expression of this protein tyrosine kinase of CLL cells (Matrai 2005). Generally, patients harbouring few or no *IGHV* mutations and with many CD38+ or Zap-70+ cells have a particularly aggressive course, whilst observations to the contrary predict indolent disease (Chiorazzi, Rai et al. 2005).

The karyotype of CLL cells can be assessed by performing fluorescence *in situ* hybridisation (FISH) on mononuclear cells obtained from peripheral blood samples. This enables chromosomal abnormalities to be detected. None of these offer any diagnostic benefit, but several abnormalities carry prognostic significance. Approximately 80% of CLL cases show aberrations in a small group of chromosomal regions (Dohner, Stilgenbauer et al. 2000). There are five common chromosomal abnormalities in CLL. 13q14 deletions are the most

common in CLL and occur in around 50% of patients (Fitchett, Griffiths et al. 1987): Patients with this isolated deletion of 13q14 have a good prognosis (Hamblin 2007). The other commonly occurring chromosomal abnormalities in CLL are deletions at 17p13 *(TP53),* 11q22-q23 *(ATM)*, and 6q21 (17p and 11q being the most severe), as well as Trisomy 12 (Hamblin 2007). Deletions of 17p are found in approximately 10% of CLL patients and the region deleted always includes the *TP53* gene that encodes p53 protein (Zenz, Mertens et al. 2010). p53 is an essential mediator of chemotherapy-induced apoptosis that will be considered in more detail in Section 1.3.2. Up to 25% of patients with advanced disease have deletions around 11q23, which is the region containing the *ATM* gene (Zenz, Mertens et al. 2010). The *ATM* gene is involved in mediating cellular responses to DNA damage. Trisomy 12 has little effect on clinical outcome (Zenz, Mertens et al. 2010), but it is detectable in up to 25% of cases (Oscier, Matutes et al. 1997). Of these chromosomal abnormalities, only loss of *TP53* provides reliable prognostic information. However, both *TP53* dysfunction and short telomere length do correlate with shorter time to first treatment in early stage CLL (Furman 2010). Although it is possible to classify patients as being at low, intermediate or high risk of disease progression, there is not yet sufficient evidence that these genetic aberrations should influence the timing of initial therapy in individual patients. Despite a lack of unifying mutations in early CLL, patients have been shown to develop genetic lesions over time (Shanafelt, Witzig et al. 2006), thus increasing their importance in the clinical course of CLL.

1.2 CLL cell development

1.2.1 Natural history

CLL cells are considered to be activated, antigen experienced B cells (Damle, Ghiotto et al. 2002). The majority of CLL clones express IgM and IgD, but up to 20% are class switched to IgG or IgA. The normal counterpart to the CLL cell remains controversial. Normal CD5+ B cells (B-1), marginal zone B cells, and memory B cells have all been proposed to be "the normal counterpart" to the CLL cell (Caligaris-Cappio, Ghia 2007). However, the natural history of CLL development remains unclear. Surface phenotype and genetic homogeneity between CLL clones of different patients (Moreau, Matutes et al. 1997, Klein, Tu et al. 2001) suggests shared mechanisms of transformation, though CLL is heterogeneous at the level of molecular and functional features (Hamblin 2002). Despite the monoclonal nature of CLL cells, unlike in other B-cell lymphomas no unifying mutations have been identified (Chiorazzi, Rai et al. 2005). Whilst long considered a disease of apoptotic suppression, as indicated by its slow clinical progression, there is strong evidence that supports the notion that CLL clones can be turned over at up to 2% of the clone per day (Messmer, Messmer et al. 2005). Thus, the disease results from both a failure of CLL cells to die in response to normal physiological stimuli, and from support of proliferation. As CLL cells die readily *in vitro*, B cell receptor stimulation and a range of other microenvironmental signals are hypothesised to contribute to the initiation and perpetuation of CLL *in vivo*.

1.2.2 The role of antigen in CLL development and progression

The B cell response to antigen is mediated through the BCR of both normal and malignant cells. The repertoire of this antigen receptor is increased by the somatic hypermutation process in germinal centres, as well as in marginal zones and around lymphoid follicles (William, Euler et al. 2002), after an antigen of adequate affinity engages the B cell receptor. This affinity maturation is the process by which naive B cells develop into long-lived memory B cells or plasma cells (Wabl, Cascalho et al. 1999). Descendent cells with increased affinity for antigen are selected to proliferate whilst the less useful cells are usually eliminated (Kelsoe 1994) via loss of antigen stimulation and critical survival signals.

Antigen stimulation is considered a prerequisite for CLL development regardless of *IGHV* gene mutations (Keating, Chiorazzi et al. 2003). Furthermore, CLL cells overexpress activation markers and underexpress markers that are downregulated by activation. Compared with age-matched control B cells, CLL cells overexpress CD23, CD25, CD69 and CD71, and have lower expression levels of CD22, CD79b and Ig, consistent with cellular activation (Chiorazzi, Ferrarini 2003). BCR surface expression is usually weak in CLL, and these low levels of BCR are indicative of anergy caused by chronic stimulation (Stevenson, Caligaris-Cappio 2004). B lymphocytes can either progress through the cell cycle or die once signal transduction is initiated by the BCR. In the ~50% of CLL cases that can transmit BCR signals to the nucleus, stimulation by BCR cross-linking can either cause or prevent apoptosis, dependent on the balance between IgM and IgD (Zupo, Cutrona et al. 2002, Bernal, Pastore et al. 2001). In the other ~50% of

cases, studies have shown activation of signalling pathways indicative of anergic B cells (Muzio, Apollonio et al. 2008). BCR signalling differs between M- and UM-clones, with M-CLL being shown to be less responsive than UM-CLL to IgM cross-linking (Zenz, Mertens et al. 2010). This may contribute to the differing clinical courses seen in these two subgroups.

Non-random use of particular *IGHV* gene segments (Kipps 1993), non-random pairing of VH and VL gene segments (Johnson, Rassenti et al. 1997), somatic hypermutation in a proportion of cases, and structural homology among the BCR of a proportion of cases (Ghiotto, Fais et al. 2004) all support the idea of CLL cells being both antigen experienced and sharing a common set of antigens (Chiorazzi, Rai et al. 2005). However, CLL cells frequently have polyreactive receptors, which bind multiple antigens, including autoantigens (Broker, Klajman et al. 1988, Sthoeger, Wakai et al. 1989). The cell biological effects of BCR cross-linking *ex-vivo*, biological features of CLL cells resembling normal B cells with antigenic stimulation, and the effects of accessory T cells on CLL cells all support a role for antigen in CLL cell maintenance (Efremov, Gobessi et al. 2007, Lanasa 2010).

Downstream of engagement of surface IgM, a survival program is elicited that involves activation of NF-κB NF-κB (nuclear factor-κB) (Section 1.6.3), increases in Mcl-1, Bcl-2, and Bfl-1 expression, and inhibition of caspase activity (Section 1.7.1/1.7.2) (Bernal, Pastore et al. 2001). Blocking of PI3-K, which mediates BCR signals in a non-redundant manner, can abrogate Mcl-1 induction and impair survival (Bernal, Pastore et al. 2001). Sustained activation of the BCR in CLL

cells has frequently been linked to activation of ERK and Akt kinases, which have been shown to be required for the induction of antiapoptotic proteins, including Mcl-1, Bcl-xl and XIAP (Section 1.7.2/1.7.10) (Longo, Laurenti et al. 2008). The kinases, p38 MAPK, and JNK (Section 1.6.2), as well as NF-AT, can also be influenced by BCR engagement (Efremov, Gobessi et al. 2007). Thus, it can be envisaged that the nature of the BCR and its stimulation affect clinical course in CLL via a complex signalling network that directs cellular growth and survival.

1.2.3 Tumour microenvironment in CLL

The balance of positive and negative signals delivered by the BCR, cell-to-cell contact, cytokines, and chemokines determines the fate of the CLL cell (Chiorazzi, Rai et al. 2005). The tumour microenvironment consists of malignant cells, host immune cells, stromal elements, and vascular cells that create a niche wherein signals can be transmitted through antigen presentation, cell–cell interactions, and paracrine signalling via cytokines and chemokines (Lanasa 2010). When B cells are activated the proteins on the surface are modified, which enables interactions with other cells and soluble signalling molecules, facilitating survival, proliferation, and differentiation (Chiorazzi, Rai et al. 2005). Microenvironmental signals can also affect the fate of CLL cells in the absence of BCR stimulation. The microenvironments differ in the bone marrow and secondary lymphoid organs. The former contains mesenchymal stem cells (MSC), whilst in the latter nurse-like cells (NLCs), T cells and follicular dendritic cells are purportedly present (Lanasa 2010). Microenvironmental interactions with CLL cells are complex and are the subject of ongoing investigation. Accessory cell pro-survival functions are complemented by adhesion molecules

and lymphokines (Munk Pedersen, Reed 2004). Some purported important CLL-supporting molecules are shown in Table 1.2, along with reported cell/s of origin.

Table 1.2. Microenvironmental factors and cell types that support CLL development and maintenance

Cell type	Microenvironmental factor
Nurse-like cells	CXCL12 (SDF-1), CXCL13, BAFF, APRIL, CD31
Marrow stromal cells	CXCL12 (SDF-1), CXCL13, CD31,
CD4+ T cells	CD154, IL-4, IFN-α, IFN-γ
Various	Angiogenic cytokines (VEGF, bFGF, TSP-1), Microvesicles; Microbial antigens.

(Munk Pedersen, Reed 2004, Burger, Hartmann et al. 2005, Nishio, Endo et al. 2005, Vogler, Butterworth et al. 2009, Kay, Bone et al. 2002, Ghosh, Secreto et al. 2010, Muzio, Scielzo et al. 2009, Ferlin-Bezombes, Jourdan et al. 1998, Deaglio, Vaisitti et al. 2006)

Signals such as those of Table 1.2 can tip the balance of cellular apoptotic signals in favour of cell survival. The PI3-K pathway has been shown to play a key role in CLL survival and growth (Cuni, Perez-Aciego et al. 2004). PI3-K can be activated by BCR stimulation (Section 1.2.2) and other survival signals, which can lead to Akt and NF-κB pro-survival signalling. Important kinases of CLL are explored in more detail in Section 1.6.2. PI3-K activation has been linked to protection of pro-survival and caspase inhibitor proteins (Cuni, Perez-Aciego et al. 2004). CXCL12 (SDF-1) can activate STAT3 and MAPK pathways (Burger, Hartmann et al. 2005). BAFF and APRIL can activate NF-κB and induce Mcl-1 (Nishio, Endo et al. 2005), and CD154 can activate NF-κB (Vogler, Butterworth et al. 2009). Microvesicles have been shown to stimulate the Akt/mTOR pathway (Ghosh, Secreto et al. 2010), whilst Toll-like receptors can induce NF-κB

activation and Akt under microbial antigen stimulation (Muzio, Scielzo et al. 2009). Clonal expansion is apparently supported by angiogenic cytokines, such as VEGF, and chemokines, such as SDF-1 (Chiorazzi, Rai et al. 2005). CD38 expression in CLL changes in response to contact with activated CD4(+) T cells, and identifies cells that are primed to proliferate (Patten, Buggins et al. 2008). CD31 stimulation of CD38 can induce proliferation and increase survival (Deaglio, Vaisitti et al. 2006). Downstream signalling consequences of microenvironmental stimulation include upregulation of the antiapoptotic BCL2, survivin, and MCL1 (Chiorazzi, Rai et al. 2005). IL-4, IFN-α, and IFN-γ are pro-survival molecules that are reported to support CLL cells via T-helper cells (Munk Pedersen, Reed 2004).

1.3 CLL Treatments

1.3.1 Current treatments and clinical trials

Patients requiring treatment are assessed for their ability to tolerate intensive therapies. Hence, they are divided according to fitness and *TP53* status. Patients with no *TP53* defects receive chemotherapy-based treatment, whereas those with *TP53* defects receive non-chemotherapy treatment such as alemtuzumab or glucocorticoids (GCs). Alkylators and purine analogues form the backbone of modern chemotherapy. Fludarabine has been shown to be superior to chlorambucil by the ECOG study (Rai, Peterson et al. 2000), and fludarabine + cyclophosphamide has been shown to be superior to fludarabine by the GCLLSG CLL4 trial, the US intergroup trial, and LRF CLL4 trial (Eichhorst, Busch et al. 2006, Flinn, Neuberg et al. 2007, Catovsky, Richards et al. 2007). More recently rituximab + fludarabine/ cyclophosphamide was shown to be superior to

fludarabine + cyclophosphamide alone by the GCLLSG CLL8 trial (Hallek, Fischer et al. 2010). This study showed that rituximab + fludarabine/cyclophosphamide is associated with a survival advantage compared to fludarabine/cyclophosphamide, so rituximab + fludarabine/cyclophosphamide is currently considered to be the treatment of choice for fit patients who are able to withstand the toxicity. For less fit patients, chlorambucil remains the standard treatment. A phase III randomised controlled trial has shown that bendamustine is superior to chlorambucil (Knauf, Lissichkov et al. 2009), but the results in the chlorambucil arm were very poor, possibly due to the sub-optimal chlorambucil dose regimen. In addition, ongoing trials are investigating the possible benefit of adding the anti-CD20 antibodies rituximab (GCLLSG CLL11), GA101 (GCLLSG CLL11) or ofatumumab (COMPLEMENT-1) to chlorambucil. The International Workshop on CLL define relapse as disease progression at least 6 months after achieving a CR (complete remission) or PR (partial remission), whilst refractory disease is defined as treatment failure or disease progression within 6 months of anti-leukaemic therapy. Patients who relapse and have not acquired a *TP53* abnormality can be expected to respond to a further course of their initial therapy although the PFS is usually shorter than after initial therapy and repeated courses often lead to drug resistance.

For patients with *TP53* defects, alemtuzumab (Section 1.3.2) is the treatment of choice. Recent data from the NCRI CLL206 trial and GCLLSG CLL2O trial (Stilgenbauer, Zenz 2010) suggest that combining alemtuzumab with GCs results in superior efficacy. Alemtuzumab-based therapy is also used for patients with fludarabine-refractory CLL irrespective of *TP53* status. Ofatumumab has useful activity in patients who are refractory to both fludarabine and alemtuzumab or

with bulky disease refractory to fludarabine (Wierda, Kipps et al. 2010). Other agents with therapeutic benefit in CLL include lenalidomide and oblimersen (Hallek 2009), and inhibitors of the B-cell receptor signalling pathway, including the PI3K delta inhibitor, CAL101.

1.3.2 Alemtuzumab and high-dose glucocorticoids

Though most patients respond to first-line therapy relapse is inevitable, and not all achieve complete remission. Patients with deletion or mutation of *TP53* at 17p13 are very unlikely to respond well to chemotherapy (Grever, Lucas et al. 2007). This is unsurprising as p53 mediates the efficacy of a wide range of anti-neoplastic agents (Wattel, Preudhomme et al. 1994) by acting as a molecular switch that blocks proliferation or initiates apoptosis. Chemo-immunotherapy, alemtuzumab, or high-dose corticosteroids are all effective as initial therapy for patients with loss of functional p53 (Grever, Lucas et al. 2007). Within recent years, monoclonal antibodies have been added to HDMP (high-dose methylprednisolone) regimen (Castro, Sandoval-Sus et al. 2008, Castro, James et al. 2009). Alemtuzumab is an anti-CD52 monoclonal antibody (MAb) that fixes complement and depletes normal lymphocytes and lymphoma cells (Hale, Bright et al. 1983). The use of alemtuzumab alone or in combination with high dose steroids results in an improved overall-response rate (ORR) and PFS, but the results are not as good as seen in previously untreated patients with a *TP53* abnormality. Despite the apparent success of alemtuzumab in clinical trials (Lozanski, Heerema et al. 2004), and effectiveness in *TP53* defective patients (Osuji, Del Giudice et al. 2005), as well as patients that do not respond to standard chemotherapy (Stilgenbauer, Dohner 2002), it is not particularly effective against nodal disease, with only 12% of all patients with lymph nodes ≥5cm responding

(Grever, Lucas et al. 2007). However, when combined with HDMP, patients with widespread cytopenias and bulky lymph nodes can be effectively treated (Pettitt, Matutes et al. 2006). Treatment options for patients who fail alemtuzumab-based therapy are limited but active agents include ofatumumab, lenalidomide, flavopiridol and high-dose glucocorticoids (GCs). The choice of therapy depends on patient fitness, previous treatment and drug availability. Patients with high-risk CLL who are not fit enough for alemtuzumab may benefit from conventional-dose GCs. GCs can also be used to treat chlorambucil-refractory CLL.

Malignant cells from patients with relapsed/refractory CLL have been demonstrated to be particularly sensitive to HDMP *in vitro*, and patient treatment response has been shown to correlate with this sensitivity (Bosanquet, McCann et al. 1995). Two subsequent studies at the Royal Marsden underlined this observation (Thornton, Hamblin et al. 1999, Thornton, Matutes et al. 2003). One study of HDMP given at a dose of $1gm/m^2/d$ each month was performed in 25 patients, 15 of whom were fludarabine refractory. An ORR of 70% was achieved with a median duration of 12 months. Responses were seen in five of ten patients with loss or mutation of the *TP53* gene. Overall, responses were seen in about half of the patients with fludarabine-refractory CLL regardless of p53 status (Thornton, Matutes et al. 2003).

GCs remain an important component of CLL therapy not least because GCs have few acute side effects, lack carcinogenic activity, and induce relatively specific cell death (Clarke, Purdie et al. 1993). Despite the usefulness of GCs in haematological malignancies, some tumours do not respond to GCs particularly

well, whilst others often develop resistance during therapy (Kaspers, Pieters et al. 1994, Moalli, Rosen 1994). Therefore, GC resistance in CLL is of clinical importance.

1.3.3 Alternatives to alemtuzumab and high-dose glucocorticoids

As well as alternative monoclonal antibody therapies to alemtuzumab, there are other treatments that do not rely upon a functional p53 pathway. Re-infusion of autologous CLL cells transduced with CD40L has received attention as a potential treatment (Wierda, Cantwell et al. 2000). As well as upregulating immune accessory molecules and bystander cells, this approach is thought to increase sensitivity to activators of the extrinsic death pathway, regardless of p53 status. Flavopiridol is a cyclin-dependent kinase (CDK) inhibitor that has been shown to be effective in killing CLL cells *in vitro* (Byrd, Shinn et al. 1998). Early trials suggested little clinical benefit, but improvements to administration schedules allowed sufficient plasma concentrations to be achieved, and clinical benefits have been reported. A phase-1 trial in advanced CLL led to an ORR of 42% with a median duration of response of over 12 months (Byrd, Lin et al. 2007). Studies are ongoing, though side effects necessitate thorough patient monitoring. R-roscovitine, which is under clinical evaluation, has been shown to be CLL cell biased in its killing specificity, and is reportedly able to induce killing in p53-aberrant samples (Alvi, Austen et al. 2005). Brefeldin, and Jasmonates are further examples of compounds that are not dependent upon the integrity of the p53-pathway in order to elicit a therapeutic response (Grever, Lucas et al. 2007).

Autologous stem cell transplant is now feasible in some patients and has low treatment-related mortality (Gribben 2009). However, there is no evidence to suggest that autologous transplantation is curative in CLL. A randomised trial that compared autologous transplantation with watch and wait, in patients who achieved CR, very good PR or nodular PR after first or second-line induction therapy (Michallet, Dreger et al. 2011), indicated that whilst autologous transplantation halved the relapse risk it had no impact on overall survival. Data regarding allogeneic stem cell transplant is more encouraging. A retrospective study by the European Group for Blood and Marrow Transplantation identified that allogeneic hematopoietic stem-cell transplantation (HSCT) is a viable treatment for chronic lymphocytic leukaemia with 17p deletion (Schetelig, van Biezen et al. 2008). Though there are few late relapses, myeloablative allogeneic HSCT is associated with high treatment-related morbidity and mortality, and this approach is also only applicable to a small number of patients (Gribben, Hosing et al. 2011). The major focus of HSCT in CLL has been reduced-intensity conditioning (RIC) allogeneic HSCT, which is applicable to the age distribution of CLL and which exploits the graft-versus-lymphoma effect (Gribben, Hosing et al. 2011). Recent data from the European Group for Blood and Marrow Transplantation suggest that the outcomes following transplants from fully matched unrelated donors are identical to sibling donors, which should increase the potential donor pool (Michallet, Sobh et al. 2010). Despite recent progress in this field, patient selection and treatment timing is under scrutiny. Furthermore, considerable morbidity and mortality of the RIC-HSCT, and high incidence of chronic extensive graft-versus-host disease mean that stem cell transplant research is still an area that requires much development.

1.4 Glucocorticoids and glucocorticoid-induced killing

1.4.1 Glucocorticoids in normal physiology

GCs are a major class of steroid hormones characterised by their ability to bind to and activate the Glucocorticoid Receptor (GR) (Chrousos, Kino 2005), which is a member of the nuclear receptor superfamily (Laudet, Hanni et al. 1992). The GR is a transcription factor that binds free GC in the cytoplasm and then translocates to the nucleus and binds to Glucocorticoid Response Elements (GREs) and negative Glucocorticoid Response Elements (nGRES), as well as other transcription factors and co-activators (De Bosscher, Vanden Berghe et al. 2003) (Section 1.5). *In vivo*, GCs are synthesised from cholesterol by a series of cytochrome P450-catalysed reactions within the adrenal cortex (De Bosscher, Vanden Berghe et al. 2003). Cortisol (hydrocortisone when synthetically manufactured), the major physiological GC, is synthesised by and secreted from the adrenal glands (Greenstein, Ghias et al. 2002). This secretion is dictated by the balance of adrenocorticotropin, and corticotropin releasing hormone, which are secreted from the anterior pituitary gland, and hypothalamus during stress, respectively, in a pulsatile and circadian way (Balsalobre, Brown et al. 2000, Reichardt, Schutz 1998). Endogenous GCs and some synthetic GCs bind to transcortin (CBG), which serves to regulate exposure (Garrel 1996). Most synthetic versions also bind other plasma proteins, with varying binding specificities (Breuner, Orchinik 2002). In the liver GCs are quickly metabolised by conjugation with a sulphate group or glucuronic acid to be secreted in the urine. Free cortisol enters cells freely due to its lipophilic properties, and can be deactivated inside cells by conversion to cortisone by type 2 11β-hydroxysteroid dehydrogenase (Yang, Zhang 2004).

The molecular structure of cortisol and the three predominant therapeutic GCs—dexamethasone (Dex), methylprednisolone (Mep), and prednisone—can be seen in Figure 1.1. Dex possesses a fluorine atom, which increases the affinity of Dex for its receptor.

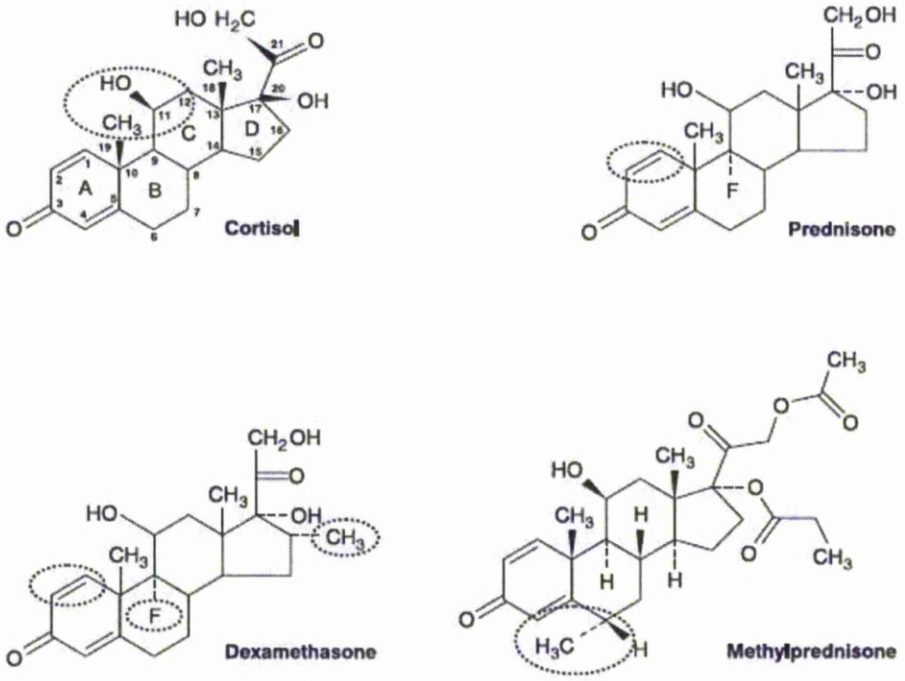


Figure 1.1. The molecular structure of cortisol and three therapeutically administered GCs (Taken from (Longui 2007)**). Methylprednisolone is labelled by the alternative name "Methylprednisone".**

GC signalling is implicated in nearly every molecular, cellular, and physiological network (Chrousos, Kino 2005), and so is a key co-ordinator of human biological processes. Whilst influencing the central nervous system, cardiovascular tone, and renal function, GCs are also critical to the activity and character of immune and inflammatory reactions (Chrousos, Kino 2005). GCs also impact on the reactions that underlie intermediary metabolism (Chrousos, Kino 2005). GCs can increase the expression of crucial enzymes of gluconeogenesis, particularly in the

liver, when the body is in the fasted state. Concomitantly, they mobilise amino acids from extra-hepatic tissues, inhibit glucose uptake in muscle and adipose tissue, and stimulate fat breakdown in adipose tissue.

1.4.2 Anti-inflammatory action of glucocorticoids

Inflammatory pathways are characterised by positive feedback loops and redundancy (Rhen, Cidlowski 2005). As a result of their immunosuppressive effects, GCs are used to treat inflammatory conditions as well as autoimmune diseases and lymphomas (Rhen, Cidlowski 2005). GCs affect immune responses in a number of ways, with effects varying according to cell type and developmental stage. GCs affect cortisol regulation by negatively feeding back on the pituitary gland (Rhen, Cidlowski 2005). In support of this, suppression of the hypothalamo-pituitary-adrenal axis can occur when GCs are used at high dose, which can lead to "steroid withdrawal syndrome" (AMATRUDA, HOLLINGSWORTH et al. 1960). GCs suppress multiple inflammatory genes either by GR-mediated repression of other transcription factors such as NF-κB and AP-1 (activator protein-1) (Section 1.6.6), or by transcriptionally increasing expression of anti-inflammatory cytokines (Section 1.6.1) or signalling proteins such as MAPK-phosphatase and IκB (Barnes 2006) (Section 1.6.2/1.6.6). GCs also inhibit the recruitment of cells to sites of inflammation by suppressing the production of chemotactic mediators, such as prostaglandins, leukotrienes, and adhesion molecules, and by inhibiting cell survival (Barnes 2006).

1.4.3 Apoptosis and necrosis

GC-induced killing displays many hall-marks of apoptosis (Greenstein, Ghias et al. 2002). Apoptosis is an orderly process of cell death that is critical for homeostasis in multicellular organisms. Defects in this process can contribute to autoimmunity, degenerative disorders, and cancer development (Strasser, O'Connor et al. 2000). Many chemotherapeutic agents are reliant on inducing this process. Apoptosis can be distinguished from necrosis by the presence of a number of features, including proteasome and endonuclease activation, condensation of chromatin, a reduction of cell volume, plasma membrane blebbing, cytoskeletal disruption, compaction of organelles, and dilation of the endoplastic reticulum (Wang, Malone et al. 2003a, Cohen 1997). In necrosis, cells swell, cell membranes and organelles are disrupted early in a hap-hazard fashion, and there is no apparent condensation of chromatin or cytoplasmic blebbing. The latter mode of cell death is potentially very damaging to the organism via toxicity and chronic inflammation, and so the execution of apoptosis is important for the maintenance of homeostasis (Savill 1997).

Apoptotic signalling can be initiated by extrinsic activation by ligand binding of death receptors, or by events within the cell leading to mitochondrial permeability transition (Green, Reed 1998). The latter event leads to release of cytochrome c and SMAC/DIABLO (Greenstein, Ghias et al. 2002). Cytochrome c positively regulates the activation of Apaf-1 and activator caspase-9 (cysteine protease-9), whilst SMAC suppresses the inhibitory effect of IAPs on survivin, caspase-9 and caspase-3 (Ferri, Kroemer 2001). In the presence of sufficient ATP this leads to caspase cleaveage of various cellular targets by the effector caspases, including

caspase-3, via a caspase cascade (Cohen 1997). Apoptosis-inducing factor (AIF) and endonuclease G are also released from mitochondria following mitochondrial membrane disruption. AIF contributes to DNA fragmentation and chromatin condensation (Susin, Lorenzo et al. 1999), and can induce caspase-independent cell death (Daugas, Susin et al. 2000). Endonuclease G is also involved in DNA fragmentation during apoptosis (van Loo, Schotte et al. 2001). Phosphatidylserine (PS) is a membrane protein that is exposed on the surface of apoptotic cells by virtue of a conformational change. *In vivo*, the exposure of PS on the surface of the plasma membrane leads to phagocytosis by neighbouring cells with PS-receptors (Fadok, Voelker et al. 1992).

Downstream components of the apoptotic signalling network are constitutively expressed and primed for activation in most cell types (Thompson 1995) (Section 1.7). Therefore, survival signals are required to repress the cell killing machinery. The functional importance of caspases in cell death is controversial, as caspase inhibition shifts the morphology of death from apoptosis to alternative pathways in a variety of models (Kroemer, Martin 2005). Moreover, caspase inhibitors only delay GC-mediated cell death and do not alter long-term survival (Kofler 2000). This is believed to be due to redundancy between caspase activation and mitochondrial damage that cannot be reversed. ATP plays an important role in apoptosis, as ATP-depleted cells have been shown to die by necrosis unless ATP generation is restored (Leist, Single et al. 1997).

1.4.4 Glucocorticoid-induced killing

Most of the reported effects of GC at therapeutic levels are mediated by the GC receptor (Schmidt, Rainer et al. 2004), and intact receptor is required for GC-induced cell killing (Greenstein, Ghias et al. 2002). Numerous models have been proposed and studies completed regarding the mechanisms that lead to the tipping of the survival balance in GC-treated lymphoid cells, particularly in ALL (acute lymphoblastic leukaemia) cells, which are particularly sensitive to GC treatment. Some studies of GC action in CLL cells have also been undertaken. Historically, GC-induced apoptosis was divided into: initiation via GR activation and subsequent gene regulation; decision making, which requires engagement of pro-survival and proapoptotic factors at the mitochondrial level; and execution, which involves caspases and endonucleases (Frankfurt, Rosen 2004). Two schools of thought had developed by 2002 regarding GC-induced killing in that it results from the expression of death inducing genes or it results from the repression of transcription factors that promote survival (Greenstein, Ghias et al. 2002). It now appears more likely that elements of both mechanisms act to kill GC-sensitive cells. It has further been proposed that two major pathways of GC-induced cell death might exist, resulting in apoptotic or necrotic morphologies, depending on the availability of the apoptotic machinery (Schmidt, Rainer et al. 2004). The former would result from regulation of typical apoptosis genes such as *Bcl-2* family members, whilst the latter from stress and detrimental effects on essential cellular functions, such as metabolism, and pH and volume control. More recently, in ALL cells, autophagy has been shown to occur as a result of Dex treatment, upstream of apoptosis (Laane, Tamm et al. 2009). This mechanism of cell death is not as well characterised as apoptosis, but was shown to be dependent

on Beclin-1, which is a protein that interacts directly with PI3-K. Regardless of how GCs kill leukaemic cells, GC-induced apoptosis can be blocked by cycloheximide and actinomycin D in the human leukaemia cell line, CEM-C7, which suggests dependence of this process on *de novo* protein and RNA synthesis (Wang, Malone et al. 2003a). Interestingly, *M-IGHV* CLL samples have been shown to respond less well than *UM-IGHV* samples to prednisolone treatment (Aleskog, Tobin et al. 2004).

It is not clear whether changes in cellular redox, acid-base balance, or metabolism are directly involved in GC-directed CLL killing. Furthermore, whether NF-κB or AP-1 play critical roles in this signalling has received little interest in CLL. An ever-growing list of other signalling pathways that can affect the GR/GC pathway also includes the cell-cycle regulator, c-Myc, and antigen receptor cross-talk, as well as cytokine signalling (Frankfurt, Rosen 2004). The signal transduction pathways affected by GCs are numerous and complex. However, a model proposed by one group provides a simple conceptual framework to build upon (Kofler, Schmidt et al. 2003). Pre-receptor defects, GR abnormalities, and deficiencies in GR-associated proteins can be classed as upstream mechanisms of resistance, whilst defects in specific response pathways, and cross-talk with other pathways that can affect or be affected by GCs provide downstream resistance mechanisms.

1.4.5 Cross-resistance in CLL

Various laboratories have investigated *in vitro* resistance of CLL cells to a range of anti-neoplastic compounds, and correlations have been tested. Sensitivity of

CLL cells to methylprednisolone, has been shown to coincide with resistance to other drugs, such as fludarabine, cyclophosphamide, and anthracyclines (Bosanquet, McCann et al. 1995). This indicates a distinct mechanism of death induction for GCs. On the contrary, it has also been suggested that tight relationships exist between responses to drugs with very different mechanisms of action, (Kivekas, Vilpo et al. 2002) and to this end prednisolone and cyclosporin A sensitivity have been linked. Mitoxantrone is a topoisomerase type II inhibitor that requires p53 to induce cell death. Resistance to mitoxantrone has been shown to exist concurrently with steroid resistance, as well as with that of vincristine (a spindle poison and mitotic inhibitor). The former agent is an intercalating agent that acts through a range of immunosuppressive mechanisms, which include inhibition of B cell function and antibody production. Both mitoxantrone and vincristine resistance have been to some extent attributed to P-glycoprotein function, which has been implicated as a cause of Dex resistance (Bourgeois, Gruol et al. 1993). Taken together, these observations would suggest that GC resistance may result from both drug-specific and general mechanisms.

Prior treatment with chlorambucil has been shown to induce sensitivity to steroids (Bosanquet, McCann et al. 1995), suggesting that some agents may be able to prime CLL cells for GC sensitivity. Contrastingly, prior treatment of patients with anthracyclines appeared to have the opposite effect, and induced *ex vivo* resistance to steroids, and platinums (p53-dependent) (Bosanquet, Bell 1996).

1.5 Glucocorticoid receptor

1.5.1 Cellular pre-GC receptor resistance mechanisms

Resistance to GC treatment could result from insufficient plasma levels in patients due to pharmacokinetics. At the cellular level, intracellular GC levels can be reduced by over-expression of P-glycoprotein, and the multidrug resistance-associated protein (Gottesman, Fojo et al. 2002). Also upstream of receptor binding, cortisol resistance can be the result of 11β-hydroxysteroid dehydrogenase type 2 activity, which converts cortisol into active cortisone in certain cell types (Rabbitt, Lavery et al. 2002). In the absence of the above resistance mechanisms, active cellular GC binds to its target receptor, the GR.

1.5.2 Glucocorticoid receptor definition

The GR is a member of the steroid hormone superfamily (Laudet, Hanni et al. 1992). Multiple human GR proteins are generated from a single gene by alternative splicing and alternative translation initiation (Lu, Cidlowski 2004) (Section 1.5.4). The *NR3C1* gene, which encodes for the GR, is located at 5q31 (Theriault, Boyd et al. 1989). Each protein is subject to a variety of post-translational modifications (Lu, Cidlowski 2004). Thus, different cell types can contain unique GR isoform compositions, which are likely to contribute to the considerable range of cell-specific responses to GCs (Lu, Cidlowski 2004). Receptor members share a variable amino-terminal transactivation domain, a central and well-conserved DNA-binding domain, and a moderately conserved carboxy-terminal ligand binding domain that also contains activating functions (De Bosscher, Vanden Berghe et al. 2003). Whilst ligand-independent activation of the GC receptor has been shown to occur in S49 T lymphoma cells following

physiological stress (Smets, Salomons et al. 1998), the dominant model of GC action in lymphopoietic cells is based upon direct activation by GC binding to the GR.

1.5.3 Glucocorticoid receptor activation

GCs diffuse across the cytoplasmic membrane and bind to the ubiquitous cytoplasmic GR (Figure 1.2) (De Bosscher, Vanden Berghe et al. 2003). Inactive GR exists as hsp90/hsp70 bound monomers, and these chaperones are positively or negatively regulated by various co-chaperones such as immunophilins, dynein, p23, hsp40, CHIP, and Bag-1 (De Bosscher, Vanden Berghe et al. 2003). Ordered assembly of the GR complex is necessary for high affinity hormone binding (Frankfurt, Rosen 2004). Upon ligand binding the GR heterocomplex undergoes molecular rearrangement, resulting in displacement of certain chaperones (Kfir-Erenfeld, Sionov et al. 2010). The GR is then phosphorylated at multiple sites, which leads to exposure of nuclear localisation sequences (NLSs), immunophilin substitution, and dynein recruitement (De Bosscher, Vanden Berghe et al. 2003). According to immunofluorescence and fractionation studies, GR-hsp90-FKBP52-dynein complex transposes to the nucleus, where GR can dimerise to yield the homodimeric DNA-binding form (Davies, Ning et al. 2002, Dittmar, Demady et al. 1997). Alternatively, it has been proposed that the GR dimerises prior to nuclear translocation, following phosporylation (Davies, Ning et al. 2002, Bledsoe, Montana et al. 2002)(Kfir-Erenfeld, Sionov et al. 2010). Co-chaperones can alter the regulatory functions of the receptors in the nucleus, as well as ligand binding, by dynamic (dis)assembly of various transcription complexes (Morimoto 2002). Activated GR binds to specific DNA sequences—GREs—as a homodimer

(Frankfurt, Rosen 2004), and then communicates with the basal transcription machinery (Yamamoto, Alberts 1976). This is known as transactivation. The dimeric GR transactivates via interaction with nuclear receptor coactivators, and other chromatin remodelling complexes and components of the transcription machinery as well as with GREs (Chrousos, Kino 2005). As well as positively regulating genes, the GR can negatively regulate genes by interaction with a negative GRE (nGRE) site (cis-repression), or by direct or indirect interference with the transcriptional activity of other transcription factors, such as NF-κB, AP-1, CREB, C/EBP, STATs, p53, and Smad (De Bosscher, Vanden Berghe et al. 2003) (transrepression) (Section 1.6.6).

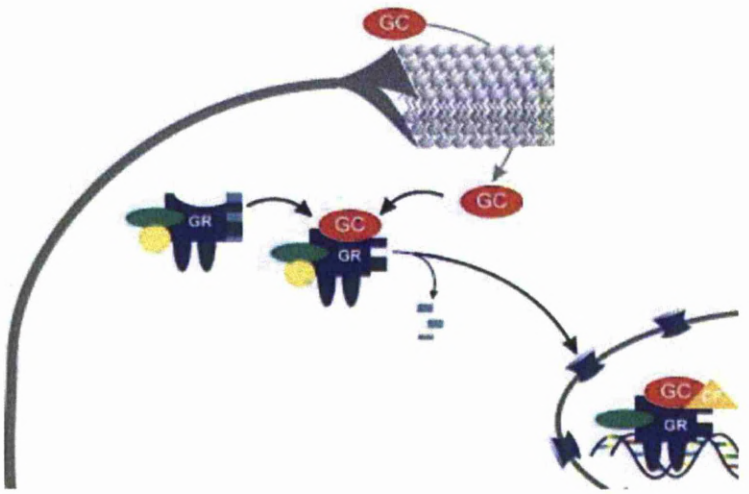


Figure 1.2. A simplified schematic of activation of and subsequent transactivation by the GR (adapted from Ploner, Schmidt et al. 2005). The hydrophobic glucocorticoid enters the cell passively, and then binds to free GR in the cytoplasm. Hyperphosphorylation leads to exposure of NLSs. The GR and its remaining chaperones enters the nucleus where the homodimeric GR can bind both positive and negative GREs, or other transcription factors, to regulate transcription.

1.5.4 GR variants

Multiple GR variants exist as a result of differential splicing and translation initiation. Originally, GR variants were reported as aberrant versions of the primary GR protein, with one such study showing that specific exons of the hormone binding domain were deleted in multiple myeloma (MM) cell lines (Moalli, Pillay et al. 1993). However, it is now the consensus that GR splice variants are deliberately expressed in order to tune cellular responses to GCs. Six major splice variants have been described. GRα, GRβ, GRγ, GRP/δ, GR-A, and GR-B (Kofler, Schmidt et al. 2003) GRα is the full-length version of the GR and is 777 amino acids long. GRα is able to bind cortisol, DNA, and other transcription factors and is responsible for transcription activation (Frankfurt, Rosen 2004). All other isoforms are truncated versions of GRα and possess altered hormone binding domains, nuclear localisation sequences, or transactivation domains (Figure 1.3), and thus are thought to modulate GRα activity (Oakley, Cidlowski 2011). GR composition varies substantially between cell types. For instance, differences in GR expression between the germinal centre and the mantle zone in normal follicles, and differences in expression among different types of lymphoma have been observed (Okazaki, Yamakawa et al. 2006). Whilst GRα can be hormone-activated, GRβ does not bind GCs, and can act as a dominant negative nuclear inhibitor of activated GRα (Shahidi, Vottero et al. 1999, Lewis-Tuffin, Cidlowski 2006). GRδ is detected in many tissues and has been shown to stimulate the transcriptional activity of GRα in malignant haematological cells (de Lange, Segeren et al. 2001).

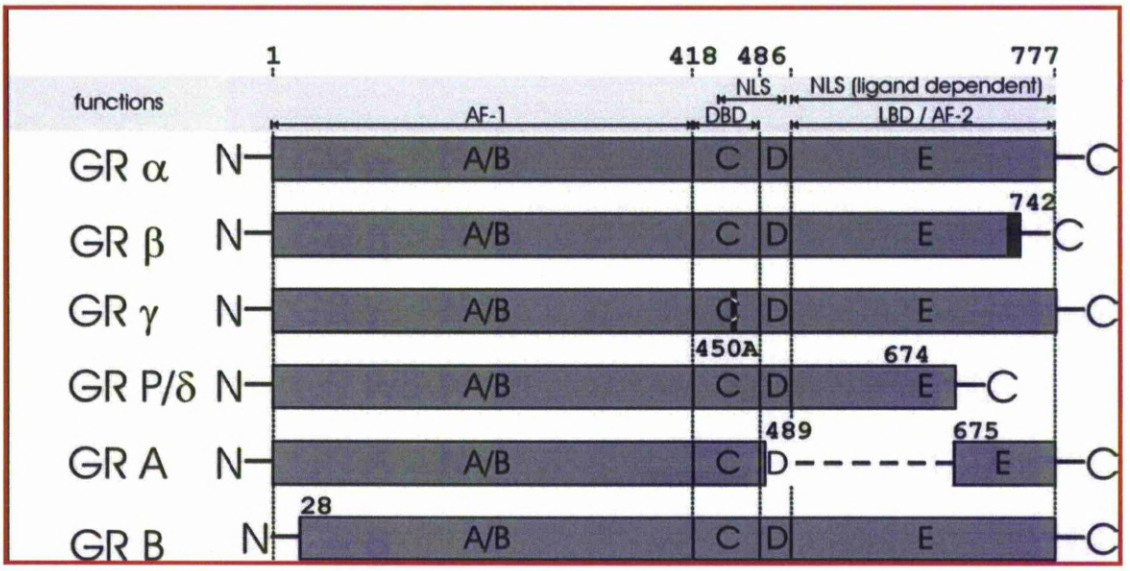


Figure 1.3. Splice variants of the GR (taken from (Kofler, Schmidt et al. 2003)**).** The bars represent GR isoforms resulting from alternative splicing, polyadenylation, and translation initiation. A/B, C, D, and E refer to specific protein regions of nuclear receptors. The numbers denote amino acids. AF, sequences implied in transactivation; NLS, nuclear localisation sequence; LBD, ligand-binding domain; DBD, DNA-binding domain.

1.5.5 GR expression levels and defects in the GR

Whether levels of GR-isoform expression in haematological malignancies are responsible for insensitivity to GCs is controversial. The sensitivity of many lymphoid cell lines to GC-induced apoptosis or growth arrest has been shown to be correlated with receptor concentration (Gehring, Mugele et al. 1984, Bourgeois, Newby 1977, Zilberman, Zafrir et al. 2004, Gruber, Carlet et al. 2009). The CEM-C1 ALL cell line is thought to owe its resistance to GCs to low GR expression, rather than downstream defects in signalling (Geley, Hartmann et al. 1996), whilst in primary ALL cells, GRβ/GRα ratios have been shown to be inversely correlated with sensitivity to GCs (Koga, Matsuzaki et al. 2005). Two other studies of ALL patient samples did not reveal a correlation between *GRβ*

mRNA levels and GC resistance (Tissing, Lauten et al. 2005, Haarman, Kaspers et al. 2004). In one early study using CLL cells, GR levels were shown not to correlate with *in vitro* sensitivity to GC, and it was also demonstrated that GR expression in circulating CLL cells did not correlate with antitumor response (Levine, Peterson et al. 1985). Contrastingly, a subsequent study demonstrated that a sample from a GC resistant CLL patient showed a markedly reduced GRα expression (~1/2) relative to untransformed lymphocytes, along with high GRβ expression (Shahidi, Vottero et al. 1999). These observations correlated with an approximate 10-fold reduction in binding affinity. GR levels have been shown to be influenced by cytokines. Pro-inflammatory cytokines can facilitate the accumulation of dominant negative GRβ, which is proposed as a mechanism of GC resistance development (Webster, Oakley et al. 2001).

GR expression levels have been shown to change over time. Most cell types show negative autoregulation of GR expression in response to GCs. However, in T cells and other cell types sensitive to GC-induced death, positive autoregulation is common (Eisen, Elsasser et al. 1988). This is unsurprising given that a GRE sequence has been detected in an untranslated exon of the GR (Breslin, Geng et al. 2001). One study demonstrated that upregulation of GR expression was required to induce apoptosis in GC sensitive T cells, and that such autoregulation may be required to facilitate a response of sufficient intensity/duration to induce killing (Ramdas, Liu et al. 1999). Positive autoregulation of GR expression has also been demonstrated in human leukemic CEM cells (Ashraf, Kunapuli et al. 1991), GC-sensitive S49 cells (Barrett, Vig et al. 1996), and a GC-sensitive myeloma cell line (Gomi, Moriwaki et al. 1990). A more recent study of CEM-C7 cells

demonstrated that the GR can positively regulate its own expression at the level of both *GR* mRNA transcription and inhibition of GR destabilisation by GCs (Pedersen, Geng et al. 2004). The *B cell translocation 1* gene is a GC-response gene and was recently shown to promote GR auto-induction in ALL (van Galen, Kuiper et al. 2010). Despite the evidence for auto-induction in various leukemic cell types, *GRα* transcript expression has been shown to fall in response to Dex in CLL cells (Meyers, Taverna et al. 2007), though GC-induced GR downregulation has been associated with GC resistance (Gross, Lu et al. 2009).

Mutant versions of GRα with decreased affinity for ligand binding, delayed translocation, and abnormal interaction with GR-interacting protein-1 coactivator have been observed (Charmandari, Kino et al. 2004). *GR* mutations have been linked to GC resistance in CCRF-CEM leukaemic cell lines (Hala, Hartmann et al. 1996). Contrastingly, a study of 15 T-ALL cell lines concluded that resistance could not be attributed to mutations in *GR* or variation in expression levels (Beesley, Weller et al. 2009). In keeping with these findings, it has also been demonstrated that the GR of leukaemia cells harvested from 6 Dex-resistant ALL xenografts possessed no defects in ligand-induced translocation and binding to a consensus GRE (Bachmann, Gorman et al. 2005). Moreover, defects in the GR are rarely encountered in primary ALL samples (Beesley, Weller et al. 2009, Irving, Minto et al. 2005, Tissing, Meijerink et al. 2005). However, polymorphisms of the GR have been correlated with reduced event-free survival in ALL patients (Labuda, Gahier et al. 2010). In CLL cells, it has been observed that a GC resistant CLL patient sample possessed ~10-fold lower binding affinity for Dex, relative to non-malignant lymphocytes from the same patient, and that *in*

vitro apoptosis induction by Dex was delayed (Shahidi, Vottero et al. 1999). However, an earlier study of CLL cells from GC-treated patients revealed an absence of DNA- and steroid-binding site mutations (Soufi, Kaiser et al. 1995).

1.5.6 Post-translational modifications of the GR

Cochaperones can inhibit GCs from binding to the GR, and affect the regulatory functions of the receptors by dynamic (dis)assembly of various transcription complexes (De Bosscher, Vanden Berghe et al. 2003). Though the GR is activated via hyperphosphorylation, each GR isoform is also subject to post-translational modifications that further modulate receptor activity. GR protein may be modified by phosphorylation, ubiquitination, and sumoylation, which may affect ligand binding, translocation to the nucleus, *trans*-activating efficacy, protein-protein interactions, or recruitment of cofactors (Ismaili, Garabedian 2004). Phosphorylation has been studied most extensively. Phosphorylation of GRα can both up- and downregulate its transcriptional activity in a gene specific manner. Also, differences at the level of cofactor recruitment, GRα half-life, and cellular trafficking have been observed in response to phosphorylation at various sites (Oakley, Cidlowski 2011). Most phosphorylation sites of the GR are located within the primary transactivation domain, AF-1 (Figure 1.3), and the basal GR phosphorylation depends upon the expression and activity of protein kinases within the cell (Kfir-Erenfeld, Sionov et al. 2010). GR nuclear-cytoplasmic shuttling and transcriptional activity are regulated through phosphorylation by MAPKs (p38-MAPK, JNK, ERK), CDKs, and glycogen synthase kinase-3 (GSK-3) (Oakley, Cidlowski 2011), as well as interactions with 14-3-3 proteins and heat shock proteins (Chrousos, Kino 2005).

Evidence for other post-translational modification of the GR comes from studies of non-haematological cell types. A role for the ubiquitin-proteasome pathway in regulating GR protein turnover, which acts to restrict transcriptional signalling by GCs, has been observed (Wallace, Cidlowski 2001). Additionally, GR sumoylation has been shown to regulate the stability of the protein as well as its transcriptional activity (Le Drean, Mincheneau et al. 2002). More recently, acetylation of the GR in response to GC treatment has been demonstrated to impair GR antagonism for NF-κB (Ito, Yamamura et al. 2006). The GR has also been shown to interact with extracellular signals via Rho family Guanine nucleotide exchange factor (Kino, Souvatzoglou et al. 2006).

1.5.7 GR-mediated alterations in gene expression

There is an emerging consensus that an interactive network of genes undergoes altered expression before the onset of apoptosis in GC-induced killing. Several comparative expression microarray studies have been applied to identify the spectrum of genes regulated by GCs in both normal lymphocytes and leukaemic cells. Approximately 20% of the human leukocyte genome has been indicated to be regulated by GCs either directly or indirectly (Galon, Franchimont et al. 2002).

One microarray compilation study has suggested that from over 10,000 genes or Expressed Sequence Tags (ESTs) at least 284 genes are directly regulated by the GR, with 70% induced and 30% repressed, though identities vary between studies (Wang, Malone et al. 2003a). In this particular comparison, a diverse set of gene changes evolved with the onset of death in two well characterised murine

lymphoma cell lines. These changes involved the pro-survival c-Myc, and NF-κB, and an increase in *Bim* expression, which was also found to be induced in CEM-C7 cells, and primary murine thymocytes. Later studies by this group uncovered *dig2* (Wang, Malone et al. 2003b), which appears to have a pro-survival function, in WEHI7.2 cells; and thioredoxin-interacting protein (*TXNIP*) as a GC-regulated primary response gene that is involved but not essential in induction of apoptosis in WEHI7.2 cells (Wang, Rong et al. 2006). A separate study of human lymphoid CEM cells that involved screening of 12,600 genes showed that a resistant sub-clone up/downregulated 88 genes prior to the onset of death that did not change in the GC-sensitive clones, suggesting that broad-acting metabolic switches may lead to a resistant phenotype (Medh, Webb et al. 2003). A concomitant analysis demonstrated differential expression between 12h and 20h, and showed a number of genes that were basally higher in resistant than sensitive clones (Webb, Miller et al. 2003). A follow up publication revealed that the Dex-sensitive clones shared the regulation of a limited set of genes, whilst the apoptosis-resistant clone showed Dex effects on a largely different set of genes. (Thompson, Webb et al. 2004). Another study of ALL cell lines identified the repression of a notable cluster of transcription, mRNA splicing and protein synthesis genes (Obexer, Certa et al. 2001). In a further ALL screen, cells from 13 patients (3h, 8h) were examined (Tissing, den Boer et al. 2007). Approximately 50% of the differentially expressed genes were functionally categorised by three major groups; MAPK pathways, NF-κB signalling, and carbohydrate metabolism, which emphasises the broad-ranging effects of GCs on the transcriptome. A further study in ALL of GC sensitive and resistant ALL cell lines, and mouse thymocytes, (6h, 24h) argues against many of the genes of

interest from model-based studies, and identified a few novel candidates for GC resistance in ALL (Schmidt, Rainer et al. 2006). These included a key regulator of glucose metabolism, a putative transcription factor, and a protein kinase implicated in cell-cycle regulation.

In MM cells Dex has been demonstrated to trigger early induction of cell repair machinery, followed by induction of pro-death and anti-survival genes (Chauhan, Auclair et al. 2002). A study of the MM cell-line MM1R uncovered a strong correlation between induction of GRE transactivation and induction of apoptosis using a GRE reporter assay (Sharma, Lichtenstein 2008). GC-sensitive human pre-B leukaemia 697 cells have also been monitored (3h, 6h, 24h, 48h) by oligonucleotide array to identify which genes are altered by GC treatment. Of 12,000 genes, 93 were induced and 28 were repressed, including genes that block proliferation, cause growth arrest, and regulate apoptosis (Yoshida, Miyashita et al. 2002).

In a broad and relatively recent study, microarray technology was used to determine whether several types of haematological cells, all sensitive to GC-evoked apoptosis, would identify a common set of regulated genes (Miller, Komak et al. 2007). Small sets of genes that correlate with GC sensitivity in cells from several haematological malignancies were identified, and some of these are also regulated in normal mouse thymocytes. Several genes in the set shared by all the GC-sensitive leukemic cells tested have been demonstrated to be important for GC-dependent apoptosis, including *BCL2L11/Bim*, *DDIT4*, *DSCR1*, *TXNIP*, *NFKBIA/IκBα*, *FKBP5/FKBP51*, and *TSC22D3/DSIPI/GILZ*. IκBα, GILZ , and Bim will be discussed in more detail (Section 1.6.3/1.6.5/1.7). *DSCR1* and

FKBP5 products antagonise the biological effects of the phosphatase calcineurin (Tissing, den Boer et al. 2007, Fuentes, Genesca et al. 2000). *TXNIP* and *DDIT4* products regulate the redox state within the cell (Chung, Jeon et al. 2006, Shoshani, Faerman et al. 2002). Other GC-responsive genes implicated in GC-mediated growth arrest, immunosuppression, and apoptosis include the serine protease *granzyme A*, the tumour suppressor gene zinc finger and BTB domain containing 16 (*ZBTB16*), the G-protein-coupled receptor T-cell death-associated gene 8 (*TDAG8*), tumour necrosis factor α-induced protein (*TNFAIP8*), the cell cycle inhibitors *p19* and *p57*, and the MAPK dual specificity phosphatase 1 (*DUSP1*) (Section 1.6.6) (Kfir-Erenfeld, Sionov et al. 2010).

1.6 Glucocorticoid receptor cross-talk

1.6.1 GR-dependent transrepression

GCs possess potent anti-inflammatory activity, but an nGRE cannot be detected in the majority of anti-inflammatory genes. In fact, transcriptional interference has been found to mostly result from cross-talk between the GR and other transcription factors (Vanden Berghe, De Bosscher et al. 2002, Herrlich, Gottlicher 2002). The GR interacts with a number of cytokine-induced transcription factors and members of the nuclear hormone receptor superfamily, via physical inhibition, disruption of the basal transcription machinery, competition for coactivators, and chromatin remodelling (Kfir-Erenfeld, Sionov et al. 2010). These include NF-κB, AP-1, NF-AT (nuclear factor of activated T-cells), CREB (cAMP response element binding protein), STATs (signal transducers and activators of transcription), Oct (octamer-binding transcription factor), IRF3 (interferon regulatory factor 3), and the T-cell specific transcription

factor GATA-3 (Refojo, Liberman et al. 2001, Gottlicher, Heck et al. 1998, De Bosscher, Haegeman 2009). Important genes repressed by GCs include cytokines, chemokines, *p27*, *c-Myc*, and *hexokinase* (Kfir-Erenfeld, Sionov et al. 2010).

1.6.2 NF-κB

NF-κB and AP-1 warrant particular interest in the context of GCs due to a well-reported antagonism with the GR (De Bosscher, Vanden Berghe et al. 2003). However, in a study of CLL cells neither baseline nor prednisolone-induced changes in AP-1 promoter binding capacity of AP-1 correlated with GC sensitivity (Bailey, Hall et al. 2001). There is conflicting evidence regarding whether GR-NF-κB antagonism is critical for GC-induced killing. For example, expression of a GR mutant in the Jurkat T-cell line that could transrepress but lacked transactivation function killed as effectively as wild-type GR in response to GC (Sionov, Spokoini et al. 2008), suggesting that suppression of survival factors by GR can be sufficient to induce killing in some contexts. Contrastingly, in CEM lymphoblastic T-cells, transrepression has been shown to not be required for GC-induced death (Tao, Williams-Skipp et al. 2001). NF-κB activity is higher in unstimulated CLL cells than in normal B cells, and has been related to *in vitro* survival (Furman, Asgary et al. 2000, Hewamana, Alghazal et al. 2008). Furthermore, the NF-κB pathway inhibitors BAY11-7082 and Kamebakaurin have been shown to accelerate apoptosis in ~70% of CLL cases, which did not involve changes in the expression of Bcl-2 or Mcl-1 (Pickering, de Mel et al. 2007). Crucially, NF-κB has been shown to be downregulated in response to Dex in CLL cells (Furman, Asgary et al. 2000). Taken together the latter observations

suggest both a key role for NF-κB in CLL cell survival, and a link between GCs and NF-κB.

The transcription factor NF-κB regulates many physiological processes, including immune regulation, inflammation, proliferation and apoptosis (Neumann, Naumann 2007). The NF-κB family of transcription factors is characterised by the 300 amino acid Rel homology domain (De Bosscher, Vanden Berghe et al. 2000). In humans, there are five well characterised subunits of NF-κB: p65 (Rel-A), p50 (NF-κB1), p52 (NF-κB2), Rel-B, and c-Rel (Ghosh, May et al. 1998). p65:p50 heterodimers are the most common form in B lymphocytes, whilst p50 homodimers, which lack classical transcription activation domains, can obstruct NF-κB-driven transactivation (Sen 2006). p65, p50, and c-Rel DNA binding has been consistently demonstrated in CLL, and p65 has been strongly linked with *in vitro* survival of CLL cells, as well as disease progression (Furman, Asgary et al. 2000, Hewamana, Alghazal et al. 2008). Therefore, these Rel-famliy proteins can be considered the principle members of the family in CLL. A physical interaction between the GR and p65 and p50 has been demonstrated *in vitro*, though whilst p65 can reciprocally repress GR activity, both p50 and c-Rel cannot (Scheinman, Gualberto et al. 1995, Wissink, van Heerde et al. 1997).

NF-κB influences CLL survival by activation of cytokines, their receptors, chemotactic proteins, and adhesion molecules, as well as by direct transcription of survival-related targets (De Bosscher, Vanden Berghe et al. 2000). NF-κB signalling is highly complex, though some of its more notable pro-survival, growth and proliferation target genes include: *Bcl-xl, Bcl-2A1, Bcl-2, c-FLIP, Bcl-*

w XIAP, c-IAP, c-Myc, c-Myb, Cyclins D1 and D2, and the cytokines IL-2, IL-6, and CD40L (Horie, Watanabe et al. 2006, Vallabhapurapu, Karin 2009). NF-κB can inhibit the AP-1 antagonist JNK via the MAPK pathway (Papa, Zazzeroni et al. 2004), and can also suppress cytochrome-c release from mitochondria through activation of Bcl-2A1 in HT1080I cells (Wang, Guttridge et al. 1999).

1.6.3 Regulation of NF-κB activity

The central mechanism underlying NF-κB regulation is the signal-induced proteolytic degradation of the family of IκBs (Neumann, Naumann 2007). This mechanism, known as the canonical pathway of NF-κB activation, requires phosphorylation of IκB by IKKs (IκB kinases). This frees NF-κB from IκB sequestration and facilitates translocation of NF-κB to the nucleus where it can exert its effects on transcription (Pickering, de Mel et al. 2007). NF-κB is subject to an autoregulatory negative feedback loop as it activates the promoter of IκBα (De Bosscher, Vanden Berghe et al. 2003). Additionally, inhibitory post-translational modification, especially phosphorylation, of Rel subunits can also occur via the non-canonical pathway (Neumann, Naumann 2007). In normal B cells both the canonical and non-canonical pathways (Figure 1.5) operate (Sen 2006). p65 and p50 are specifically activated by the canonical pathway. Further, p65 can also be selectively sequestered within the nucleolus, and dimer exchange has also been reported (Neumann, Naumann 2007).

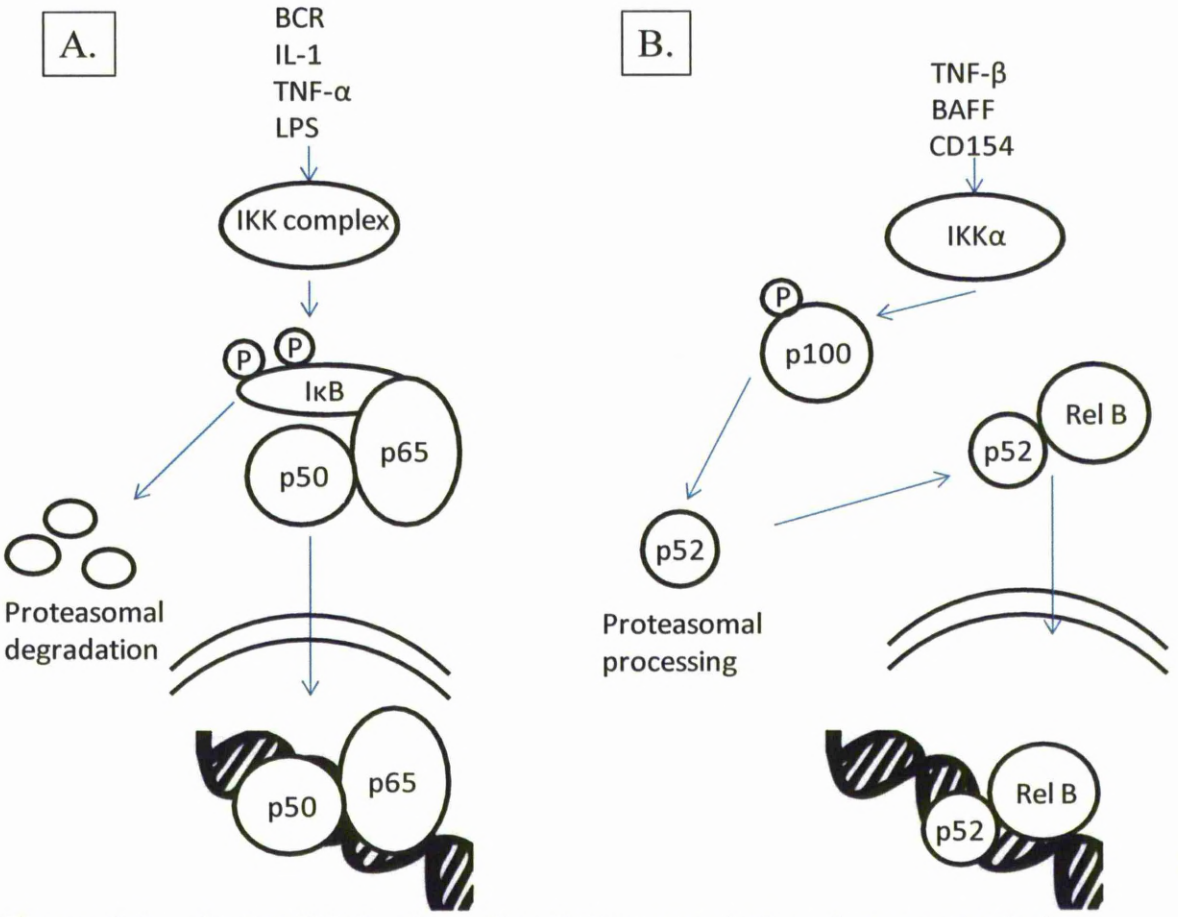


Figure 1.4. A simplified schematic of the canonical and non-canonical pathways of NF-κB regulation in B lymphocytes (adapted from (Sen 2006)**).** A. The canonical pathway can be activated by a number of extracellular stimuli, which activate IKKs that phosphorylate p65-bound IκBs. The p65/p50 heterodimer is then released from cytoplasmic sequestration and translocates to the nucleus to elicit signalling alterations via transactivation and transrepression. B. The non-canonical pathway selectively activates IKKα, which phosphorylates the precursor protein, p100. Following processing into mature p52, the p52-Rel-B heterodimer forms and enters the nucleus.

NF-κB activation is downregulated during CLL cell culture, and so *in vivo* signals are strongly implicated in its regulation (Bernal, Pastore et al. 2001, Furman, Asgary et al. 2000). NF-κB is subject to phosphoregulation by various pathways, including p38, ERK, JNK, PKA, PKC, PI3-K, and Ras, which can affect localisation, dimerisation, translocation, DNA binding, stability, transactivation, and cofactor recruitment (De Bosscher, Vanden Berghe et al. 2003). BCR

stimulation and Akt activation increases NF-κB pro-survival activity via IκB degradation (Barragan, Bellosillo et al. 2002). NF-κB activity in CLL is also modulated by a variety of cytokines. Most CLL cells express CD40, which can be activated by CD40-ligand (CD154) via cell-cell contact. CD154 signalling augments NF-κB activation (Furman, Asgary et al. 2000). BAFF (B-cell activating factor) has also been shown to increase NF-κB activity (Barragan, Bellosillo et al. 2002, Claudio, Brown et al. 2002). Notch receptor and ligand expression is involved in survival and apoptosis resistance in CLL cells, and is associated with increased NF-κB activity, along with c-IAP2 and XIAP expression (Rosati, Sabatini et al. 2009). Downstream, GSK-3β prevents epigenetic silencing of NF-κB-mediated gene transcription and thus maintains cell viability and prosurvival Bcl-2 and XIAP expression in CLL cells, (Ougolkov, Bone et al. 2007).

1.6.4 GR-mediated-transrepression of NF-κB

A number of research groups have demonstrated a direct physical association between GR and p65 *in vitro*, and *in vivo* (Scheinman, Gualberto et al. 1995, Nissen, Yamamoto 2000, Ray, Zhang et al. 1995, Adcock, Nasuhara et al. 1999)(De Bosscher, Vanden Berghe et al. 2003). Several models have been proposed to describe the interaction of GR and NF-κB. These include: competition for shared promoters; mutual masking of transactivation domains; induction of post-translational modifications; and competition for limited coactivator proteins (De Bosscher, Vanden Berghe et al. 2003, Greenstein, Ghias et al. 2002). The latter mechanism is not strongly supported in the case of transrepression, and struggles with a lack of specificity (De Bosscher, Vanden

Berghe et al. 2001, Sheppard, Phelps et al. 1998). There is also evidence of tethering, in which activated GR blocks recruitment of the basal transcription machinery (Nissen, Yamamoto 2000). In ALL cells the NF-κB antagonist tumour necrosis factor α-induced protein 3 (TNFAIP3) has been shown to be upregulated by GCs (Tissing, den Boer et al. 2007). GCs have also been reported to induce IκBα synthesis (Auphan, DiDonato et al. 1995, Scheinman, Cogswell et al. 1995) and thus downregulate NF-κB indirectly, though this observation was highly cell-type dependent. Contrastingly, in the presence of the protein synthesis inhibitor cycloheximide GCs have still been shown to inhibit NF-κB in some cell types (De Bosscher, Schmitz et al. 1997, Wissink, van Heerde et al. 1998), and GC analogues exist that upregulate IκBα but do not inhibit NF-κB activity (Heck, Bender et al. 1997). Taken together the latter two observations support a mechanism involving a more direct relationship between the GR and NF-κB.

1.6.5 GILZ

The GR can influence NF-κB by inducing GILZ (D'Adamio, Zollo et al. 1997). The human GILZ promoter has 6 GREs. GILZ has been demonstrated to interfere with NF-κB-mediated transcription in T cells, and to participate in GC-induced thymocyte apoptosis (Ayroldi, Migliorati et al. 2001)(Delfino, Agostini et al. 2004). Furthermore, in mesenchymal stem cells, GILZ is able to block NF-κB nuclear translocation (Yang, Zhang et al. 2008). It is expressed in resting B lymphocytes, within which it is downregulated when they are activated (Glynne, Ghandour et al. 2000). GILZ is characterised by a leucine zipper domain, which is a well-conserved protein motif that acts as a DNA-binding site. There are four main isoforms that vary in expression between cell types (Soundararajan, Wang et

al. 2007). GILZ can modulate a variety of cellular functions, including sodium channel activity and proliferation (Soundararajan, Wang et al. 2007). For instance, in mouse T cells, GILZ diminishes the activity of Ras and Raf targets, including ERK1/2 and Akt. Silencing of GILZ in these cells inhibited antiproliferative effects of GC (Ayroldi, Zollo et al. 2007). GILZ regulation has implications for broader survival signalling. It can suppress MKP-1 activity, which in turn can suppress the MAPKs p38 MAP-kinase, ERK, and JNK. Furthermore, GILZ has been shown to prevent binding of AP-1 to its target DNA and can inhibit activation-induced events in Jurkat T cells (Mittelstadt, Ashwell 2001). GILZ has also been reported to promote nuclear exclusion of the Foxo3, which can inhibit Bim and p27 upregulation (Latre de Late, Pepin et al. 2010). As well as being induced by the GR, GILZ can be regulated by PI3-kinase/AKT signalling, which contributes to GILZ induction in MM cells (Grugan, Ma et al. 2008).

1.6.6 The kinome

GR receptor stability, localisation, interactions and transcriptional targets can be regulated by kinase activity (Section 1.5.6). Furthermore, GC treatment can lead to phosphorylation of the GR by MAPKs, GSK, and CDKs (Sionov, Spokoini et al. 2008)(Kassel, Sancono et al. 2001). Protein kinases, including MEK/ERK, PI3-K/Akt and m-TOR, can antagonise GC-induced apoptosis of haematopoietic cells, (Miller, Garza et al. 2007).

The mitogen-activated protein kinases, ERK, JNK, and p38 phosphorylate and activate transcription factors that promote proliferation and inflammation, such as

NF-κB and AP-1 (Clark, Lasa 2003) (Section 1.6.2), via parallel kinase cascades. Opposingly, GR activation is predominantly anti-inflammatory (Rogatsky, Logan et al. 1998). Of the three pathways, ERK usually provides pro-survival signals, whilst JNK and p38 favour apoptosis (Xia, Dickens et al. 1995). ERK, p38, and JNK are inactivated via GC-mediated upregulation of MAPK phosphatase-1 (MKP-1) in many cell types (Abraham, Clark 2006, Horsch, de Wet et al. 2007). As GILZ can be upregulated by GCs (Section 1.6.5), and can mediate ERK signalling in this context (Ayroldi, Zollo et al. 2007), induction of the *GILZ* and *MKP-1* genes provides a mechanism by which GCs can modulate all three of the major MAPK pathways. Bim induction (Section 1.7) by GC in an ALL cell line has been shown to depend on the activation of p38 MAP kinase (Lu, Quearry et al. 2006), which may be attributable to Foxo3a activation (Cai, Xia 2008). However, p38 inhibition has been shown to not affect GC-induced apoptosis of highly GC sensitive thymocytes and thymic lymphoma cells (Spokoini, Kfir-Erenfeld et al. 2010).

GSK3 has been shown to play a role in GC-induced apoptosis in an MM cell line as well as in T cells (Spokoini, Kfir-Erenfeld et al. 2010). GSK3 is a serine/threonine kinase that acts on a variety of susbstrates including metabolic enzymes and transcription factors, including NF-κB. It has two main isoforms; α and β. Unlike most kinases it is active in resting cells (Galliher-Beckley, Williams et al. 2008). Several other kinases inhibit GSK, including Akt protein kinase, cAMP-dependent protein kinase A (PKA), and serum and GC-induced kinase SGK1 (Kfir-Erenfeld, Sionov et al. 2010). Phosphorylation of Ser_{404} of the GR by active GSK3β can protect against Dex killing in U-2 osteoblast cells, in

part due to retention of the GR in the cytosol (Galliher-Beckley, Williams et al. 2008). In a T cell line, GC has been shown to increase GSK3β activity via phosphorylation of Ser_9 (Spokoini, Kfir-Erenfeld et al. 2010). GC has been demonstrated to displace GSK3α from the GR in GC-sensitive thymocytes and thymic lymphoma cells, leading to GSK3α/β inhibition of Bim and GC-induced killing (Spokoini, Kfir-Erenfeld et al. 2010). Conversely, GSK3 inhibition prevents GC-mediated Bim upregulation in follicular lymphoma cells (Nuutinen, Ropponen et al. 2009).

PI3-K can also play a role in GC sensitivity of lymphoid cells (Section 1.2.3). For instance, inhibition of PI3-K but not that of ERK or PKCδ can enhance Dex-induced apoptosis in Follicular Lymphoma cells (Nuutinen, Postila et al. 2006). PI3-K activation has been shown to prevent downregulation of the downstream pro-survival proteins: Bcl-xl, FLIPL, and XIAP (Cuni, Perez-Aciego et al. 2004). The PK (protein kinase) family has also been reported to be involved in GC-induced killing of lymphoid cells. Calcium-independent PKC activation has been shown to be involved in GC-induced apoptosis of thymocytes (Asada, Zhao et al. 1998), whilst in CLL PKA has been demonstrated to be required for GC-induced apoptosis by modulating GR-mediated signal transduction (Tiwari, Dong et al. 2005). Moreover, in CLL cells inhibition of PI3-K or PKC has been shown to increase Dex-induced apoptosis in the presence of survival factors (Barragan, Bellosillo et al. 2002). GC action has been linked to interaction with the activating T-cell receptor-associated Src-family kinases, including Lck, Lyk, and Fyn in T cells (Ghosh, Baatar et al. 2009, Lowenberg, Tuynman et al. 2005), which have been shown to antagonise GC-induced killing (Iwata, Hanaoka et al.

1991). Though predominantly a kinase of T cell origin, *Lck* mRNA expression has been positively correlated with Dex resistance in CLL cells, and its inhibition has been shown to enhance GC sensitivity of CLL cells (Harr, Caimi et al. 2010).

CDK- and MAPK-mediated phosphorylation of the GR exert opposing effects on transcriptional activity. Thus, pathways affecting cell proliferation can affect the response to GCs. In support of this idea, G_0/G_1 arrested ALL cells show increased sensitivity to GCs (Obexer, Hagenbuchner et al. 2009). GCs have been shown to act upon various regulators of cell cycle progression. For instance, CDK2 is crucial for GC-induced apoptosis of thymocytes (Frankfurt, Rosen 2004). Downregulation of c-Myc accompanies apoptosis by GCs in CEM-C7 cells, and sustained expression of c-Myc provides protection against GC-induced killing (Thompson 1998). Contrastingly, cyclin D3 and c-Myc have been shown to induce cell cycle arrest but not apoptosis in CCRF-CEM lymphoblastic leukaemia cells (Ausserlechner, Obexer et al. 2004). The proteasome is a protease complex located in the cytoplasm and nucleus that degrades polyubiquitinylated proteins (Ciechanover 1994). The proteasome has been implicated in GC-induced killing by proteasome inhibition experiments (Vugmeyster, Borodovsky et al. 2002). Recognized targets of the proteasome include transcription factors that regulate genes necessary for cell proliferation (*c-Fos*), enzymes whose activity is crucial for cell proliferation (*ornithine decarboxylase*), and cell cycle regulatory proteins ($p27^{Kip1}$) (Frankfurt, Rosen 2004). Thus, the proteasome may influence GC-induced killing via effects at the level of cell-cycle regulation.

1.6.7 Inhibitor of apoptosis proteins (IAPs)

NF-κB has been shown to transactivate the antiapoptotic IAPs. IAPs are involved in cell division, apoptosis, or survival pathways (LaCasse, Mahoney et al. 2008). IAPs have been shown to inhibit caspases-3, -7, and -9 (Salvesen, Duckett 2002), and thus modulate apoptosis. SMAC/Diablo physically inhibits IAPs following mitochondrial perturbation (Du, Fang et al. 2000), and IAPs are able to catalyse their own ubiquitination for degradation by the proteasome (Yang, Fang et al. 2000). IAPs are potentially relevant to this study as they are elevated in CLL relative to other leukaemias and lymphomas (Schattner 2002). It has also been shown that *c-IAP2* and *XIAP*, as well as *Bcl-2* are elevated at mRNA level by more than one fold in CLL relative to a panel of lymphoid malignancies (de Graaf, van Krieken et al. 2005). Moreover, the IAP family of proteins can suppress apoptosis induced by a range of triggers, including GCs, and overexpression of XIAP can lead to an accumulation of apoptotic resistant immature T cells (Conte, Liston et al. 2001). The main role of IAPs in suppression of apoptosis is to antagonise the extrinsic death receptor pathway. This pathway depends on caspases for the initiation of apoptosis. Despite the less essential role of caspases in the intrinsic pathway, IAP activity may impact upon killing via this pathway. IAPs inhibit the activation of effector caspases, have been correlated with poor prognosis, and show elevated expression in haematological malignancies (Fulda 2009). XIAP is the most strongly supported member of the family as possessing marked anti-caspase activity (Eckelman, Salvesen et al. 2006).

1.6.8 Cytokines

Cytokine signalling affects survival, as well as differentiation and proliferation. Most cytokines signal through the Jak-STAT pathway, which is dependent upon cytokine binding to cell surface receptors, activation of STAT proteins, and alterations in target genes (Rogatsky, Ivashkiv 2006). STAT proteins act in concert with other transcription factors and cofactors, which can both up- and downregulate their activity. There is evidence of an interaction of pro-inflammatory STATs with the anti-inflammatory GR (Rogatsky, Ivashkiv 2006). Several cytokines have been shown to counteract GC-induced killing, including those expressed by thymic epithelial cells (Gao, Kinoshita et al. 1996). IL-6 and IFN-α inhibit GC-induced apoptosis in myeloma cells (Ferlin-Bezombes, Jourdan et al. 1998, Chauhan, Pandey et al. 1997). Also, IL-6, IL-4, and IL-9 have been shown to inhibit GC-induced killing of thymocytes (Van Snick, Houssiau et al. 1996, Bauer, Liu et al. 1998). IL-15 inhibits GC-induced apoptosis in activated B cells (Bulfone-Paus, Ungureanu et al. 1997). α-IFN, IL-4, and IFN-γ can inhibit GC-induced DNA fragmentation in CLL cells (Panayiotidis, Ganeshaguru et al. 1994), and IL-4 can protect CLL cells from steroid-induced apoptosis (Mainou-Fowler, Prentice 1996). SOCS1, the suppressor of cytokine signalling, is suggested to act as an early mediator of the cross-talk between GC and cytokine signalling (Haffner, Jurgeit et al. 2008), whilst STAT-3 has been shown to be a coactivator of GR transactivation (Zhang, Jones et al. 1997). Cytokine rescue from GC-induced apoptosis in T-cells is mediated through inhibition of IκBα expression (Xie, Seward et al. 1997).

1.7 Glucocorticoids and the apoptotic balance

1.7.1 Core mechanism of the intrinsic apoptosis pathway

Apoptosis (Section 1.4.3) plays a critical role in the killing of malignant cells by chemotherapy and radiation, particularly in lymphoid cells (Johnstone, Ruefli et al. 2002, Cory, Adams 2002). The BH3-only subgroup of proteins of the Bcl-2 family particularly stands out as being important in apoptosis due to its universality (Huang, Strasser 2000). BH3-only proteins are transcriptionally activated, post-translationally modified (particularly by phosporylation), or released from sequestration in response to death stimuli (Puthalakath, Strasser 2002). Bcl-2 family proteins have distinct binding partners (Chen, Willis et al. 2005). Unsurprisingly, proapoptotic family members with restricted targets are weaker killers. The proapoptotic BH3-only Bim and Puma have been shown to potently engage all antiapoptotic members (Chen, Willis et al. 2005), emphasising their importance in particular.

The primary model of intrinsic apoptosis activation involves proapoptotic members of the family binding and antagonising antiapoptotic members (Certo, Del Gaizo Moore et al. 2006), which leads to indirect activation of the effector proteins Bax and/or Bak (Danial, Korsmeyer 2004) (Figure 1.5). In response to death and damage signals, sensitiser BH3-only proteins inhibit antiapoptotic proteins, thus preventing their repression of activator BH3-only proteins, and multi-domain proapoptotic proteins. Activator proteins can also repress multi-domain antiapoptotic proteins. As well as the direct activation model, an indirect Bax/Bak activation model has been supported by cell free studies, whereby Bax

and Bak are bound and activated directly by proapoptotic proteins (Kuwana, Bouchier-Hayes et al. 2005). Following Bax/Bak activation, pores are formed in the outer mitochondrial membrane resulting in mitochondrial depolarisation and release of cytochrome c and Smac/DIABLO, and caspase activation (Danial, Korsmeyer 2004) (Section 1.4.3). BH3-only profiling studies have been particularly illuminating in the field of Bcl-2 family research. This method has been used to determine cellular dependence on specific antiapoptotic proteins, by measuring the pattern of mitochondrial sensitivity to a panel of peptides derived from the BH3 domains of the BH3-only proteins (Certo, Del Gaizo Moore et al. 2006).

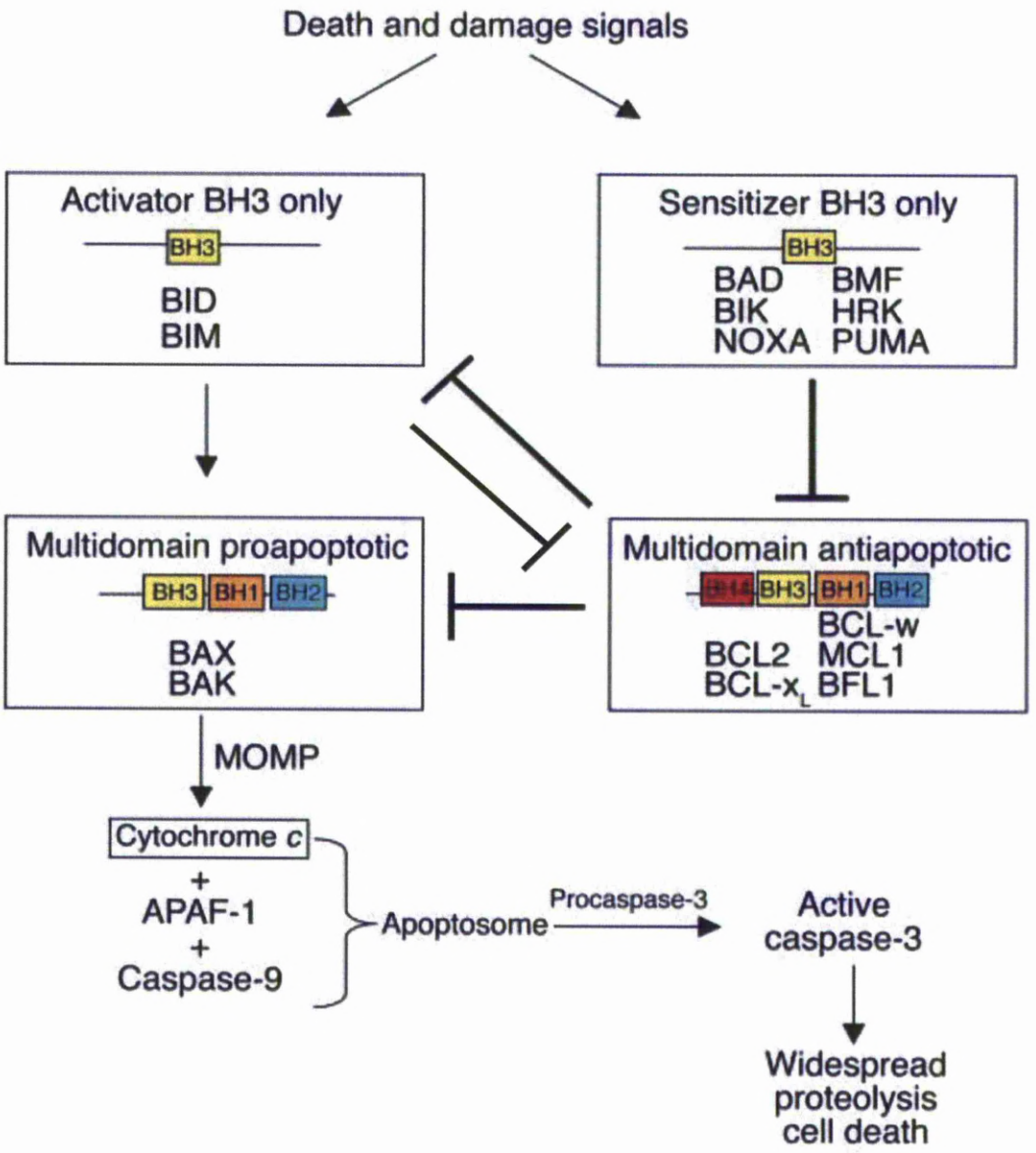


Figure 1.5. The mitochondrial cell death pathway (Adapted from (Del Gaizo Moore, Brown et al. 2007)**.** Bad, Bik, Noxa, Bmf, Hrk, and PUMA are sensitiser BH3-only proteins. Bid and Bim are activators, whilst Bax and Bak are executioners. Bcl-2, Bcl-xl, Bcl-w, Mcl-1, and Bcl2-A1 (Bfl-1) are antiapoptotic.

1.7.2 Apoptotic proteins in CLL

In CLL cells the expression of the *BCL-2* gene family is shifted towards protection from apoptosis, as Bcl-2 members are frequently overexpressed in leukaemic cells (Gottardi, Alfarano et al. 1996)(Ploner, Schmidt et al. 2005). The sensitiser BH3-only proteins Bmf and Noxa, as well as the antiapoptotic Bcl-2 are elevated in CLL cells (Mackus, Kater et al. 2005). In a prominent study of 58 CLL patient samples, the expression of apoptosis-regulating proteins was measured and compared with *in-vivo* and *in-vitro* response to chemotherapy (Kitada, Andersen et al. 1998). Several Bcl-2 family proteins, including Bcl-2, Bcl-xl, Mcl-1, Bax and Bak, BAD, BAG-1 Caspase-3 were evaluated. Of note, Bcl-2, Mcl-1, BAG-1, Bax, Bak, and Caspase-3 were commonly expressed in circulating CLL cells, whilst Bcl-xl, and Bad proteins were not. Failure to achieve complete remission was linked to Mcl-1 overexpression, which emphasises the importance of this antiapoptotic protein. The identification of BAG-1 as being partially associated with failure to achieve complete remission is interesting in the context of GC response as it is a GR-chaperone that modulates GR function (Schmidt, Wochnik et al. 2003).

1.7.3 Bcl-2 family members and GCs

Induction of proapoptotic Bcl-2 family proteins and repression of their antiapoptotic counterparts has been observed in response to GCs. Prednisone treatment has been shown to induce a decrease in Bcl-2 and Bcl-xl levels in ~50% of primary ALL cells, with Bax upregulation detected in 14/21 samples (Casale, Addeo et al. 2003). A later systematic analysis of Bcl-2 family expression, GC regulation, and function of Bcl-2 molecules in primary ALL lymphoblasts has

been performed (Ploner, Rainer et al. 2008). The prominent effects that were observed were Bmf and Bim induction, and repression of Noxa; the repression of the latter has since been shown to impair GC sensitivity (Ploner, Rainer et al. 2009). Within these investigations, knockdowns within CCRF-CEM cells emphasised the importance of Bim, and to a lesser extent Bmf, to GC-regulated apoptosis. Antiapoptotic proteins were not regulated consistently by GCs, with overexpression and knockdowns leading only to delayed death onset. Single knock-outs of proapoptotic Bim (Bouillet, Metcalf et al. 1999), and Puma or Noxa (Villunger, Michalak et al. 2003) show some resistance to GC treatment.

Further evidence supports a role for Bim as a mediator of GC cytotoxicity. In Dex-treated ALL cells, changes in the expression of *Bim*, as well as targets of c-Myc and NF-κB have been detected (Wang, Malone et al. 2003a). *Bim* mRNA and protein corresponding to all 3 major isoforms of Bim were induced over 24h in ALL cells, preceding the onset of death, and this finding was developed by immunoblotting in CEM-C7 cells and murine thymocytes. Further to this, a panel of B-ALL cell lines showed Bim protein induction to correlate with GC sensitivity, and siRNA knockdown of Bim expression was related to increased cell survival (Abrams, Robertson et al. 2004). Bim shRNA dramatically inhibited Dex-induced apoptosis in another study using a T-ALL cell line (Lu, Quearry et al. 2006). A compelling series of experiments underlined Bim as possessing particular relevance in GC-induced killing of CLL cells (Iglesias-Serret, de Frias et al. 2007). The effect of 10mM Dex (24h) on the mRNA expression of a series of death-specific proteins in 9 CLL patient samples was studied. This covered BH3-only and Bax-like proapoptotic proteins, Bcl-2-like antiapoptotic proteins,

IAP family proteins, and others. *Bim* expression almost doubled, whilst *Bad*, *Bmf*, and *Noxa* were unchanged. *Mcl-1* and *HIAP1* were repressed by approximately a quarter, and *FLIP* expression approximately doubled. Thus, *Bim* and *FLIP* induction were particularly prominent. As *FLIP* is an apoptosis-suppressing protein that blocks early events in TRAIL/TNF family death receptor signalling it is unlikely to play an important role in GC-induced killing.

Puma as well as Bim, but not Noxa, has been shown to contribute substantially to Dex-induced killing of lymphocytes of a *BCL-2* transgenic mouse (Erlacher, Michalak et al. 2005). Puma had previously been shown to be induced by Dex in thymocytes (Han, Flemington et al. 2001), and Puma deficiency can partially protect thymocytes from GC-induced killing (Villunger, Michalak et al. 2003). Further to these findings, the CDK inhibitor CDKN2A has been shown to sensitise T-ALL cells to FAS and GC-induced killing via Puma induction and Mcl-1/Bcl-2 repression. Despite this evidence, Puma is not upregulated in pre-B cells in response to Dex, whilst Bim is (Erlacher, Michalak et al. 2005). Similarly, GC does not upregulate Puma in mouse ALL xenografts (Xu, Wang et al. 2006).

Data supporting an important role for Bmf in GC-induced killing is limited. Bmf is a BH3-only Bcl-2 family member that is normally sequestered to myosin V motors by binding to the dynein light chain (DLC) 2. Certain damage signals lead to Bmf release and Bcl-2 antagonism. Loss of Bmf in mice can protect a range of B-lymphocytes against GC-induced killing (Labi, Erlacher et al. 2008). Furthermore, Bmf -/- mice develop B-cell lymphadenopathy. A further member

of the Bcl-2 family that is not strongly supported as being important in GC-induced killing is Bad. However, Bad induction has been shown to occur in thymocytes in response to GCs (Mok, Gil-Gomez et al. 1999). Also, as Bad is held in its inactive phosphorylated state by a 14-3-3 protein (Wang, Pathan et al. 1999), GR sequestration of the chaperone (Kino, Souvatzoglou et al. 2003) could hypothetically free Bad to facilitate downstream death signalling.

1.7.4 Bim

Human Bim is encoded by the *BIM* gene on chromosome 2q12-13 and consists of 6 exons including a non-translated exon. More than 18 isoforms have been reported in human cells (Adachi, Zhao et al. 2005). The three major isoforms—Bim-EL, Bim-L, and Bim-S (Figure 1.6)—are consistently observed in haematopoietic cells, including CLL (Kfir-Erenfeld, Sionov et al. 2010). Bim-S is the most potent isoform (O'Connor, Strasser et al. 1998), probably by virtue of its lack of post-translationally modifiable domains. Bim has an important role in haematopoietic cell homeostasis and responses to a wide variety of chemotherapeutic drugs, and can be upregulated in response to cellular stress, UV, and growth factor withdrawal (Bouillet, Metcalf et al. 1999). Furthermore, Bim-deficient granulocytes are resistant to spontaneous death in culture, and mice lacking Bim contain strikingly high numbers of leukocytes (Bouillet, Metcalf et al. 1999). Bim has also been shown to be critical in BCR-driven B cell apoptosis (Enders, Bouillet et al. 2003).

Bim-induced killing has been reported to involve antagonism of prosurvival Bim binding partners, but also direct Bax activation. A recent study using mice with

mutated Bim BH3 domains (Merino, Giam et al. 2009) supported this direct activation model. However, other groups have previously suggested that the direct activation model is invalid (Ewings, Wiggins et al. 2007). Bim-AD (a less well studied and less abundant isoform) and Bim-S can heterodimerise both with the death antagonists Bcl-2 and Bcl-xl, and with death-inducing Bax. Moreover, by studying a mutated version of Bim-AD that can bind Bax but not Bcl-2, it has been shown that Bim can directly activate Bax-mediated cell death (Marani, Tenev et al. 2002). With respect to the other isoforms of Bim, an indirect activation model is better supported whereby Bim displaces Bcl-2 family proteins from Bax and Bak, which leads to oligomerisation of Bax and Bak, and pore formation. A further less well supported model of proapoptotic Bim signalling purports that Bim translocation to the ER mediates ER stress signalling (Morishima, Nakanishi et al. 2004).

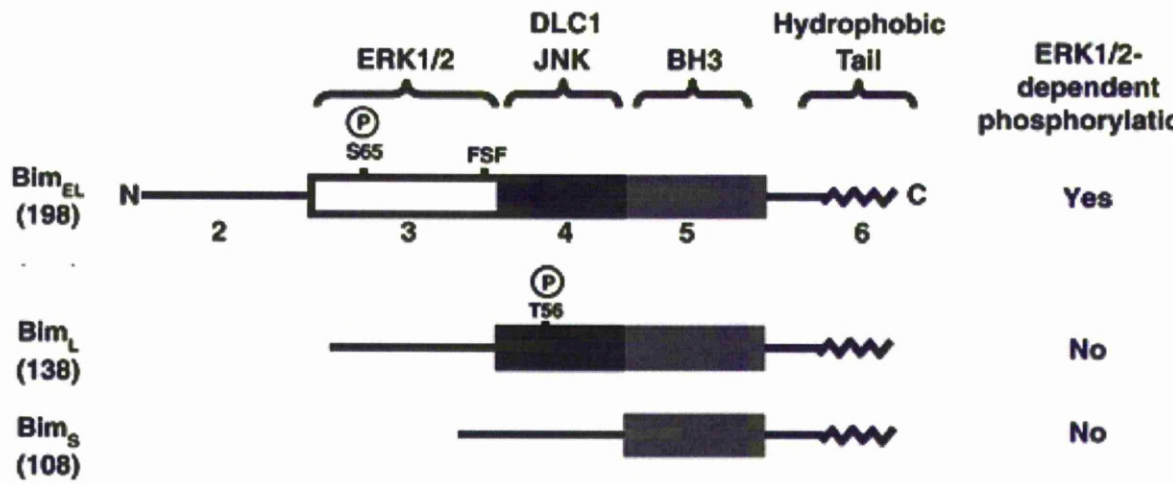


Figure 1.6 The three major Bim isoforms of CLL cells (Adapted from (Ley, Ewings et al. 2005)**).** The structure of some of the proteins arising from alternative splicing of *Bim* transcript is shown, with the BH3-only domain and the hydrophobic C-terminal highlighted. Bim-S is encoded by exons 2, 5 and 6. Bim-L is encoded by exons 2, 4, 5 and 6. Exon 4 includes a binding site for DLC1, and JNK phosphorylation sites, including Thr_{56} in Bim-L. Bim-EL is

encoded by exons 2, 3, 4, 5 and 6. Exon 3 includes an ERK1/2 docking domain (FSF) and ERK1/2 phosphorylation sites, including Ser_{69}. ERK1/2-dependent phosphorylation of Bim-EL targets it for proteasomal degradation and may prevent binding to Bax. The numbers below the Bim-EL figure represent the different exons.

1.7.5 Bim Regulation

In several studies involving intact cells, *Bim* mRNA is regulated by survival signals that act through Forkhead and Akt transcription factors. In thymocytes *Bim* mRNA can be induced as early as 30 minutes in response to GCs (Zhao, Li et al. 2007). New protein synthesis has been shown to be required for *Bim* mRNA upregulation (Abrams, Robertson et al. 2004), but other evidence suggests that protein synthesis is not required (Lu, Quearry et al. 2006). Despite the observation that Bim protein levels increase during spontaneous apoptosis in CLL cells (Iglesias-Serret, de Frias et al. 2007), mRNA levels do not appear to. The Bim promoter does not contain a classical GRE. However, signalling upstream of Bim can be directed by GCs, resulting in both transcriptional and post-translation control. Foxo3a/FKHRL1, RUNX3, and E2F1 (ER stress detector) are suggested to mediate Bim upregulation (Dijkers, Medema et al. 2000, Zhao, Tan et al. 2005, Yamamura, Lee et al. 2006). Foxo transcription factors, especially Foxo3a, have been shown to regulate Bim in various cell types (Sunters, Fernandez de Mattos et al. 2003, Urbich, Knau et al. 2005, Gilley, Coffer et al. 2003, Stahl, Dijkers et al. 2002). Importantly, siRNA knockdown of Foxo3a can significantly reduce GC-induced apoptosis in lymphocytes (Ma, Xie et al. 2008). Interestingly, Bim induction by GC in an ALL cell line has been shown to depend on the activation of p38 MAP kinase (Lu, Quearry et al. 2006).

Mere upregulation of Bim is insufficient for apoptosis in T lymphoma cells (Kfir, Sionov et al. 2007), and possibly other lymphoid cell types. Despite this finding it is unclear how GCs might activate Bim, though various post-translational modifications have been observed (Figure 1.6). Bim is expressed in healthy cells where Bim-EL and Bim-L can be maintained in an inactive form by binding to microtubules (Puthalakath, Huang et al. 1999). Release of Bim-EL and Bim-L from cytoskeletal sequestration and relief of ERK phosphorylation of Bim-EL can facilitate Bim activation (Puthalakath, Huang et al. 1999, Ley, Balmanno et al. 2003). More specifically, Bim-EL protein is targeted for ubitquitinylation and proteasomal degradation by ERK phosphorylation of Bim Ser_{69} (Fukazawa, Noguchi et al. 2004). This Bim phosphorylation may also impair interactions between Bim and Bax to prevent the oligomerisation of Bax (Ley, Ewings et al. 2005, Marani, Hancock et al. 2004). Bim release from microtubules takes place in the absence of caspase activation, and so is not simply a mechanism of apoptotic amplification (Puthalakath, Strasser 2002). Bim protein can be modulated by both survival signals and the proteasome (Iglesias-Serret, de Frias et al. 2007). Phosphorylation of Bim-L by JNK can prevent Bim sequestration by dynein (Ley, Ewings et al. 2005), whilst phosphorylation of Bim-EL has also been shown to release the apoptotic protein from microtubules, and induce apoptosis via p38-mediated Ser_{65} phosphorylation (Chen, Zhou 2004, Cai, Chang et al. 2006). p38 is required for GC-induced Bim upregulation in CEM T-ALL cells (Lu, Quearry et al. 2006) (Section 1.6.6). GSK3 may be responsible for Bim activation through a direct interaction (Spokoini, Kfir-Erenfeld et al. 2010)(Section 1.6.6).

As well as ERK, RACK1 (Receptor for Activated C-Kinase 1) and SOCS (Suppressor of Cytokine Signalling) have been shown to ubiquitinylate Bim-EL for degradation upon paclitaxel treatment of breast cancer cells, whilst Grb10 (an insulin receptor-binding protein) has been shown to inhibit Bim-L by phosphorylation in HEK294 cells (Zhang, Cheng et al. 2008, Hu, Zhang et al. 2010). Bim-EL is predominantly serine phosphorylated in murine lymphoma lymphocytes and thymocytes, which has been reported to prevent Bim-induced apoptosis (Seward, von Haller et al. 2003). Downstream of apoptosis induction, Bim-EL can be cleaved by caspases to yield a hyperactive form, with higher affinity for Bcl-2, leading to amplification of apoptotic signals (Chen, Zhou 2004).

1.7.6 Bim antagonists

A compelling study of Bim binding partners showed that Mcl-2, Bcl-XL, and Bcl-2 are pulled down with all three major Bim isoforms in co-immunoprecipitation experiments using human B cells (Gomez-Bougie, Bataille et al. 2005). DLC8 was found to associate only weakly with Bim-EL and Bim-L. Furthermore, subcellular fractionation showed predominant localisation of Bim and Bim supressors to the mitochondrial fraction. The Mcl-1/Bim complex was the most abundant of the three dominant complexes. ABT-737, a BH3-only mimetic, is a potent killer of CLL cells, and can displace proapoptotic proteins from Bcl-2, Bcl-xl, and Bcl-w (Certo, Del Gaizo Moore et al. 2006), but not from Mcl-1 or Bcl-2A1 (Oltersdorf, Elmore et al. 2005, van Delft, Wei et al. 2006). BH3-only profiling (Section 1.7.1) uncovered the importance of Bcl-2 sequestration of Bim in CLL survival (Del Gaizo Moore, Brown et al. 2007). In this study, ABT-737

antagonism was shown to be likely the result of Bim displacement from the Bcl-2/Bim complex. Moreover, a further study has shown that co-incubation of CLL samples with Dex and ABT-737 can sensitise CLL samples to the BH-3 only mimetic (Mason, Khaw et al. 2009). However, this could not be predicted by responses to either agent alone. This relationship between Bim and Bcl-2 is further supported by evidence that the two proteins can mutually effect the expression of each other. In T cells, absence of Bim causes a drop in Bcl-2 protein levels, whilst Bcl-2 overexpression leads to an increase in both Bim mRNA and protein (Jorgensen, McKee et al. 2007).

Bcl-2 protein is consistently elevated in CLL relative to normal B cells and other lymphoid cells, though the level varies significantly between cases. Overexpression of Bcl-2 in human pre-B lymphocytes and myeloma cell lines can prevent apoptosis (Alnemri, Fernandes et al. 1992), and Bcl-2 knockout mice display accelerated apoptosis of thymocytes in response to GCs (Veis, Sorenson et al. 1993). Moreover, Bcl-2 mRNA and protein (48h) has been knocked down by antisense RNA, which correlated with increased apoptosis (Pepper, Thomas et al. 1999b). Clinically, high levels of Bcl-2 have been associated with shorter overall survival in previously treated patients, and resistance to fludarabine treatment (Buggins, Pepper 2010). However, one study of *ex vivo* drug sensitivity of CLL samples showed that Bcl-2 expression did not associate with sensitivity at all (Bosanquet, Sturm et al. 2002). High Bcl-2 expression in CLL has been attributed to stimulation by IL-8 (Molica, Vitelli et al. 1999), nucleolin stabilisation of *Bcl-2* mRNA (Otake, Soundararajan et al. 2007), hypomethylation of the promoter region of the *BCL2* gene (Hanada, Delia et al. 1993), and *miR-15* and *miR-16*

deletion or downregulation (Buggins, Pepper 2010). Bcl-2 has also been reported to be upregulated by the cytokines IFN-α/γ and IL-1/IL-2, IL-4, and IL-6.

Mcl-1 confers GC resistance in various cell types (Lopez-Royuela, Balsas et al. 2010, Wei, Twomey et al. 2006, Saffar, Dragon et al. 2008). Mcl-1 has also been implicated in drug resistance as it is strongly associated with failure to achieve a complete response to chlorambucil, fludarabine, and rituximab (Buggins, Pepper 2010). Overexpression in transgenic mice predisposes to lymphomagenesis, and Mcl-1 is required for lymphoma and MM cell survival, as demonstrated by antisense ablation (Packham, Stevenson 2005). Mcl-1 expression is rapidly altered by transcription and protein stability by various environmental cues. It has a rapid turnover and can be targeted by ubiquitination for proteasomal degradation. During apoptosis, Mcl-1 is an efficient caspase substrate (Clohessy, Zhuang et al. 2004). Interestingly, caspase cleavage can turn Mcl-1 from a pro-survival molecule to proapoptotic (Michels, Johnson et al. 2005). The proapoptotic Bak associates with Mcl-1 and Bcl-xl, but not Bcl-2, Bcl-w, or A1 (Willis, Chen et al. 2005).

1.7.7 Bax and Bak

Bax is a cytosolic protein that translocates to the mitochondrion and undergoes a conformational change and oligomerisation as a critical activating step of apoptosis in CLL (Dewson, Snowden et al. 2003, Vogler, Dinsdale et al. 2008). Bak resides at the outer mitochondrial membrane (OMM) and is also activated by conformational change and oligomerisation. It has been shown that increased proteasomal degradation of Bax is a common feature of poor prognosis in CLL

(Agrawal, Liu et al. 2008), which confirms the importance of this protein in CLL cell killing. Thymocytes from double knock-out mice lacking Bax and Bak are GC resistant (Rathmell, Lindsten et al. 2002). In ALL GCs do not significantly alter Bax or Bak expression (Bachmann, Gorman et al. 2007), but do lead to their activation (Rambal, Panaguiton et al. 2009, Laane, Panaretakis et al. 2007). Moreover, using fluorescence microscopy and Western blotting, a strong correlation has been observed between the number of Dex-induced apoptotic CLL cells and the percentage of cells stained with antibodies that recognise active Bax and Bak conformation (Bellosillo, Villamor et al. 2002), as well as the number of cells displaying Bax integration into the mitochondrial membrane. Therefore, Dex-induced apoptosis is associated with changes in intracellular localisation of Bax, and conformational changes of Bax and Bak in CLL. It was also shown that caspase inhibitors do not compromise this interaction, suggesting that this process occurs upstream of caspase activation.

In ALL cell lines, the Bax-Bcl-2 ratio has been positively correlated with Dex sensitivity (Salomons, Brady et al. 1997), and high Bcl-2-Bax ratios in CLL cells may predict a drug resistance phenotype (Pepper, Thomas et al. 1999a). Contrary to these findings, in one study of 39 CLL samples and 8 drugs, reduced Bax levels did not correlate with *ex vivo* resistance to purine analogs and corticosteroids ($P>0.5$), despite a correlation existing between Bax levels and traditional therapies; anthracyclines, alkylating agents and vincristine (all $P<0.5$). (Bosanquet, Sturm et al. 2002). Thus, this study supports the existence of distinct mechanisms of GC and purine analog action from those of alkylating agents, vinca-alkaloids, and anthracyclines.

1.8 GCs and cellular distress

GCs can perturb homeostasis by transcription dependent and by non-transcription dependent mechanisms. The former includes regulation of genes involved in metabolism, oxygen radical control, calcium flux, or pH and volume control (Schmidt, Rainer et al. 2004). The latter includes rapid nongenomic effects, which have been shown to occur within minutes of GC exposure (Song, Buttgereit 2006). These include a transient increase in calcium, alterations in redox status, elevation of intracellular hydrogen peroxide and other reactive oxygen species (ROS), lysosomal release of cathepsin B, and activation of sphingomyelinase with subsequent ceramide production (Spokoini, Kfir-Erenfeld et al. 2010). In T cells GC treatment can induce transient increases in cytosolic calcium concentrations and calmodulin activation, the release of protein kinases from the GR complex, release of cathepsin B form lysosomes, and activation of sphingomyelinase to produce ceramide and sphingosine (Cifone, Migliorati et al. 1999, McConkey, Hartzell et al. 1989, Wang, Muller et al. 2006, Lepine, Lakatos et al. 2004).

An important role for alterations in metabolism and cellular homeostasis in GC-mediated killing has previously been evidenced. Inhibitors of Ca^{2+} activated proteases have been shown to inhibit thymocyte GC-induced killing (Squier, Cohen 1997). Inositol triphosphate receptor (IP3R) is a Ca^{2+} channel that can facilitate loss of calcium homeostasis and thus induce death. Lymphocytes undergoing apoptosis in response to GCs have been found to display increased expression of inositol triphosphate receptor (IP3R) (Frankfurt, Rosen 2004), and IP3R-deficient T-cells have been shown to be resistant to GC-induced apoptosis. Sphingomyelin hydrolysis to produce ceramide and sphingosine occurs following

cell membrane disruption and internalisation of sphingomyelin (Tepper, Ruurs et al. 2000), which facilitates blebbing and shedding of membrane vesicles. Ceramide can also potentiate the proapoptotic action of Bax (Pastorino, Tafani et al. 1999), and it can activate, the proapoptotic protein, Bad (Stoica, Movsesyan et al. 2003). Furthermore, ceramide can lead to release of cytochrome c and AIF (Di Paola, Zaccagnino et al. 2004). GCs can rapidly activate phosphatidyl-inositol-specific phospholipase C via PKC, and induce diacylglycerol generation, which is required for sphingomyelinase activation and ceramide production in thymocytes (Cifone, Migliorati et al. 1999). Hydorgen peroxide production, along with molecular oxygen, has been shown to contribute to GC-induced killing of thymocytes, (Tonomura, McLaughlin et al. 2003). Glutathione is an antioxidant that opposes the damaging effects of ROS, free radicals and peroxides. Dex resistance has been shown to correlate with an increase in glutathione level (Inoue, Takemura et al. 2002) in a pre-B cell line, and glutathione S-transferase expression has been associated with both T-ALL relapse and failure of GC to activate Bim and p38 in CCRF-CEM cells (Hosono, Kishi et al. 2010).

Other cellular effects of GCs that may be of importance to GC-induced killing include alterations in glucose metabolism, cell volume control, and direct mitochondrial interference by the GR. Prednisolone resistance can be modulated by inhibition of glycolysis in ALL cells (Hulleman, Kazemier et al. 2009). K^+ (potassium ions) contributes to cell volume control, and thus its regulation is important to cell homeostasis. An inhibitor of K^+ plasma membrane channels has been found to be an effective inhibitor of GC-induced apoptosis of thymocytes (Dallaporta, Marchetti et al. 1999). However, dysregulation of K^+ is a

consequence of the commitment to apoptosis, as a significant efflux of intracellular sodium and potassium occurs during apoptosis, which is thought to contribute to cell shrinkage. Contrastingly, inhibition of cation movement can actually inhibit apoptosis (Hughes, Bortner et al. 1997). Rapid and persistent translocation of the GR to mitochondria in GC-sensitive lymphoid cells in response to GC treatment has been observed (Sionov, Cohen et al. 2006, Talaber, Boldizsar et al. 2009). It has been suggested that steroid hormones can activate mitochondrial gene transcription (Demonacos, Karayanni et al. 1996, Scheller, Seibel et al. 2003), and an interaction of the GR with the redox-regulating thioredoxin has been reported (Psarra, Hermann et al. 2009). GR can also interact with the mitochondrial death-associated protein DAP3 (Hulkko, Zilliacus 2002).

1.9 Thesis Purpose and Overall Strategy

The mechanism of GC-induced killing in CLL cells is relatively undefined and little is known about resistance. A summary of reported mechanisms of GC-induced killing of haematopoietic cells is shown in Figure 1.7. The working hypothesis of this thesis is that defective pro-death/anti-survival signalling in response to GC stimulation causes insensitivity of CLL cells to GC. Thus, the aims of this thesis were to understand why some samples respond to GC treatment while others do not, identify markers of resistance, and elucidate potential strategies for restoring sensitivity of CLL cells to GCs. To achieve these aims a panel of primary CLL samples were characterised for sensitivity to GC treatment, and the most and least responsive samples were compared and contrasted to establish at what point(s) death signalling mediated by the GC receptor was blocked in the resistant samples.

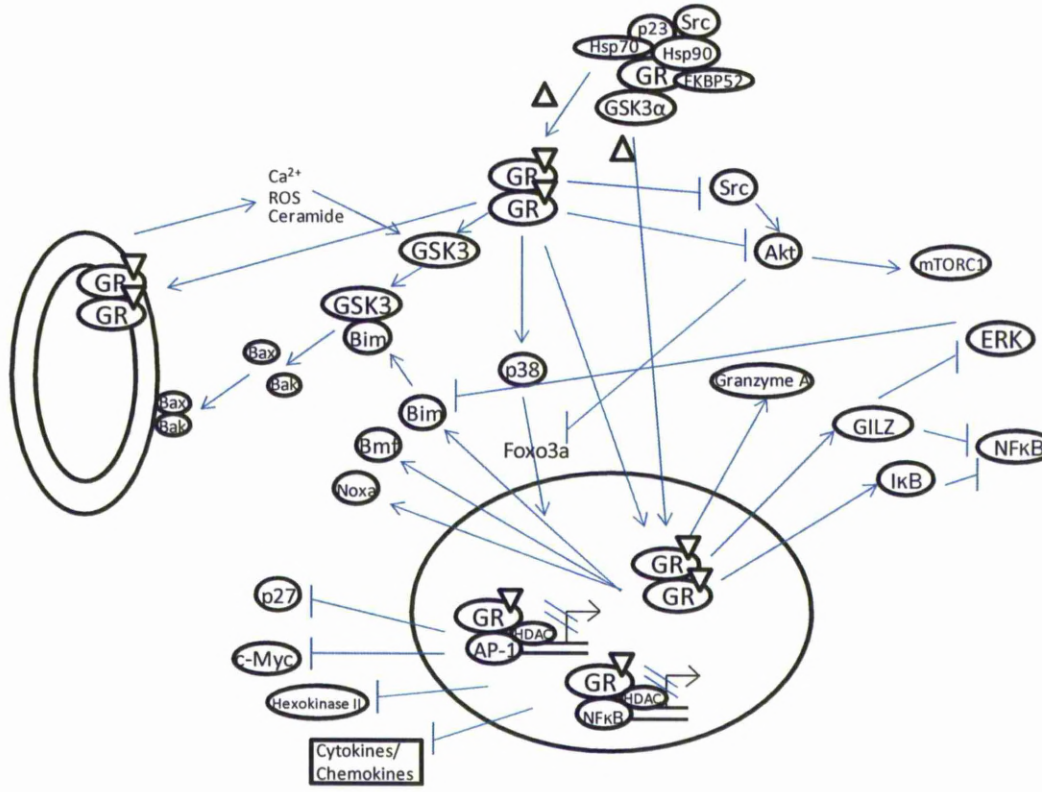


Figure 1.7 Mechanisms of GC-induced killing of haematopoietic cells (adapted from (Kfir-Erenfeld, Sionov et al. 2010)**).** GR is bound to a heat shock complex in the cytosol in the absence of ligand. Upon ligand binding the GR heterocomplex undergoes molecular rearrangement, the GR is phosphorylated at several serine residues, and either undergoes dimerisation or first translocates to the nucleus. GR has been reported to translocate to the mitochondria as well as the nucleus of GC-sensitive lymphoid cells. In the nucleus, the GR transcriptionally activates and represses a range of targets. Bim is of particular importance for the initiation of apoptosis, and is indirectly upregulated by FoxO3a, which is dependent upon p38 activity. GSK3 can affect the efficacy of Bim by phosphorylation. Bim either directly activates Bax and Bak or antagonises antiapoptotic agonists of these apoptotic effector proteins. GR inhibits prosurvival and proinflammatory gene regulation by NF-κB and AP-1, and inhibits cell cycle progression via c-Myc, amongst other targets. GR also represses NF-κB at the cytoplasmic level by inducing GILZ and IκB. GILZ upregulation can increase Bim-EL expression via ERK inhibition.

Chapter 2: Glucocorticoid-induced killing of CLL samples

2.1 Introduction

Though most CLL patients respond to first-line therapy relapse is inevitable, and not all achieve complete remission. As CLL progresses following initial therapy, the frequency of abnormal p53 function increases to nearly 50% of patients, possibly due to treatment driven mutation and subsequent clonal selection (Lozanski, Heerema et al. 2004). Those patients with deletion or mutation of *TP53* at 17p13q are very unlikely to respond well to chemotherapy (Grever, Lucas et al. 2007) (Section 1.1.3), however, alemtuzumab is effective in these patients, and recent data from the GCLLSG CLL2O trial (Stilgenbauer, Zenz 2010) suggests that combining alemtuzumab with GCs yields improved efficacy (Section 1.3.2). Furthermore, cells from patients with relapsed/refractory CLL and p53 aberrations have been demonstrated to be particularly sensitive to HDMP (Thornton, Hamblin et al. 1999, Thornton, Matutes et al. 2003). Despite the usefulness of GCs in such patients, some tumours simply do not respond to GCs (primary resistance), whilst others often develop resistance during therapy (secondary resistance) (Kaspers, Pieters et al. 1994, Moalli, Rosen 1994). Therefore, there is a pressing need to elucidate GC resistance mechanisms in CLL.

"GC resistance" can be absolute or relative; apply to all or specific effects of GCs; occur at the level of the entire organism or affect a particular cell clone; can be specific or apply to all GCs; and be reversible or irreversible (Kofler, Schmidt et al. 2003). For the purpose of this investigation, GC resistance will be defined as the relative *in vitro* insensitivity of primary CLL samples to specific GCs. *In vitro* sensitivity can be considered clinically relevant as patient treatment response has been previously shown to correlate with *in vitro* sensitivity of CLL samples to GC treatment (Bosanquet, McCann et al. 1995).

There is a substantial literature relating to the complexities of GC-induced killing of haematopoietic cells. This killing displays many hall-marks of apoptosis (Greenstein, Ghias et al. 2002) (Section 1.4.4), though alternative pathways of killing that can lead to a necrotic character have also been proposed (Schmidt, Rainer et al. 2004). Induction of proapoptotic BH3 proteins (Wang, Malone et al. 2003a, Han, Flemington et al. 2001) and repression of their antiapoptotic counterparts (Chauhan, Auclair et al. 2002, Casale, Addeo et al. 2003) has been observed in response to GCs in various haematopoietic cell types (Section 1.7). Moreover, indicators of apoptosis have frequently been observed in CLL cells in response to GC treatment (Tiwari, Dong et al. 2005, Chandra, Gilbreath et al. 1997, McConkey, Chandra 1999).

In vitro sensitivity of CLL cells to methylprednisolone has been shown to correlate with resistance to other drugs, such as fludarabine, cyclophosphamide,

and anthracyclines (Bosanquet, McCann et al. 1995), and so GC-specific signalling defects may lead to resistance (Section 1.4.5). Alternatively, a multi-drug resistance mechanism may predispose GC resistance, as *in vitro* sensitivity of CLL cells to prednisolone and cyclosporin A has been shown to correlate (Kivekas, Vilpo et al. 2002) despite these agents acting via very different mechanisms. Furthermore, resistance to mitoxantrone (p53-dependent) has also been shown to exist concurrently with steroid resistance (p53-independent), as well as with that of vincristine (Bourgeois, Gruol et al. 1993).

Rai stage, *IGHV* status, *TP53* deletion/mutation, and CD38 expression facilitate the designation of poor prognosis patients (Section 1.1.3), whilst treatment history can affect efficacy of future treatment. Prior treatment with chlorambucil has been shown to induce sensitivity to steroids (Bosanquet, McCann et al. 1995), and treatment of patients with anthracyclins can induce resistance to steroids (p53-independent) and platinums (p53-dependent) (Bosanquet, Bell 1996) (Section 1.4.5). Patients that require treatment with GCs are those with disease refractory to or relapsed from frontline chemotherapies (Section 1.3.2). Such disease often, but not always, possesses aberrations in the p53 pathway (Pettitt, Matutes et al. 2006). However, though p53 pathway aberration predicts for poor response to chemotherapy, it is not predictive of GC response (Thornton, Matutes et al. 2003). A previous study within this research group revealed a correlation between p53 dysfunction and other poor prognosis indicators, including UM-*IGHV* and expression of CD38 in CLL cells (Lin, Sherrington et al. 2002). Patients expressing UM-*IGHV* tend to show signs of more aggressive disease and have a

reduced survival compared to patients with (mutated) M-*IGHV* (Section 1.1.3). Furthermore, prednisolone resistance has been correlated with M-*IGHV* (Aleskog, Tobin et al. 2004).

Previous work from within this department has shown that CLL cells respond to varying degrees when treated with Dex *in vitro*. This GC is commonly used in the treatment of patients with lymphoproliferative disorders in continental Europe, and features regularly in the scientific literature discussed in Chapter 1. The primary aim of this chapter was to establish which CLL samples of a panel of over 50 samples were most sensitive and resistant to Dex, and to validate the methodology used to achieve this aim. This would allow selection of the most sensitive and resistant samples to be compared and contrasted.

2.2 Materials and Methods

2.2.1 CLL patients

All of the patient samples studied were from the University of Liverpool Leukaemia Bank. Samples are annotated with a range of clinical and laboratory data, including some important CLL biomarkers such as mutation status of immunoglobulin heavy chain variable gene (*IGHV*) and *TP53* gene deletion. These samples were obtained from patients who had at least 5 x 10^9 B lymphocytes/l (5000/μl) in the peripheral blood and a typical CLL immunophenotype (mature lymphocytes expressing CD19, CD5, CD23, and weak, clonally restricted surface immunoglobulin light chain) (Hallek 2009). Peripheral blood CLL samples were obtained with informed consent and with the approval of the Liverpool Research Ethics Committee.

2.2.2 Sample selection

Samples for initial screening of drug sensitivity were selected from the biobank on the basis that they were in plentiful supply (>40 vials) and possessed white blood cell count of >30x10^9/l. In this way, the samples selected were of relatively high CLL cell purity and were sufficiently abundant to be available for sequential experiments.

2.2.3 Cell isolation and storage

Peripheral blood mononuclear cells from CLL patients were prepared by centrifugation (500rcf, 30mins) over Lymphoprep (d=1.077, Nycomed, Oslo, Norway). Cells were then washed in PBS and slowly resuspended in an ice-cold

solution of RPMI (GIBCO) + 10% fetal calf serum (FCS, Biosera, East Suusex, UK) and 10% dimethyl sulphoxide (DMSO; Sigma, Poole, Dorset, UK). Cells were then aliquoted into cryotubes at 1-3 x 10^7/ml, frozen at -80°C and finally cryopreserved in liquid nitrogen.

2.2.4 Poly-HEMA coating of tissue culture plates

Poly-HEMA (poly(2-hydroxyethyl methacrylate)) is a hydrophilic, non-toxic polymer that prevents cell adhesion (Folkman, Moscona 1978). For coating of tissue culture plates, a poly-HEMA stock (120mg/ml in 95% ethanol; Sigma) was diluted to 12mg/ml in 95% ethanol and specific volumes aliquoted into the wells (30μl for 96-well plates, and 200μl for 24-well plates). The plates were then incubated for at least 48hours at 37°C to allow evaporation, until dry.

2.2.5. In-house cell culture

Cryopreserved cells (1ml) were thawed rapidly at 37°C, and resuspended slowly to a final volume of 8-12mls per vial (maximum of 2 vials per universal) with RPMI 1640 medium (GIBCO) supplemented with 10% FCS (Biosera, East Suusex, UK), 2mM L-glutamine (Invitrogen), and 100U/ml penicillin + 100μg/ml streptomycin (Invitrogen). Following centrifugation at 500rcf for 5 minutes, cells were washed in 1ml of PBS, counted, and resuspended at double concentration in the above medium. Cells were cultured at 2x10^6 cells/ml (~1x10^6 cells/cm^2), at 37°C and 5% CO_2, in poly-HEMA-precoated tissue culture plates with 96 flat-bottomed wells, in the presence or absence of cyclodextrin-encapsulated

dexamethasone (Sigma, Poole, Dorset, UK). Following incubation for 1-3 days cells were harvested, centrifuged at 500rcf for 5min, resuspended at double concentration, and then counted using either a standard manual haemocytometer and microscope, or an automatic Cellometer Auto T4 Haemocytometer (Nexcelom Bioscience, MA, USA) in Nexcelom Cell Counting Chambers.

2.2.6 Measurement of cell viability by flow cytometry

2.2.6.1 Propidium Iodide (PI) viability measurement

PI is a fluorochrome that can be used to measure cell death by flow cytometry. PI freely enters both live and dead cells but is selectively excluded by live cells (Darzynkiewicz, Bruno et al. 1992).

To analyse cell viability by FACS, cultured cells were resuspended and added to an equal volume of PI (Sigma, Poole, Dorset, UK) at 5μg/ml in PBS in a FACS tube. Following incubation on ice in the dark for 30 minutes, analysis was carried out using a FACScan flow cytometer (Becton Dickinson, Oxford, UK). Live cells appear as PI-dim, while dead cells are PI-bright. 10,000 events were collected as standard. Representative data for this method of viability measurement is shown in Figure 2.2.1.

The formula $((Viability_{(UT)}-Viability_{(T)})/Viability_{(UT)})x100$ was used to define percentage killing relative to untreated controls.

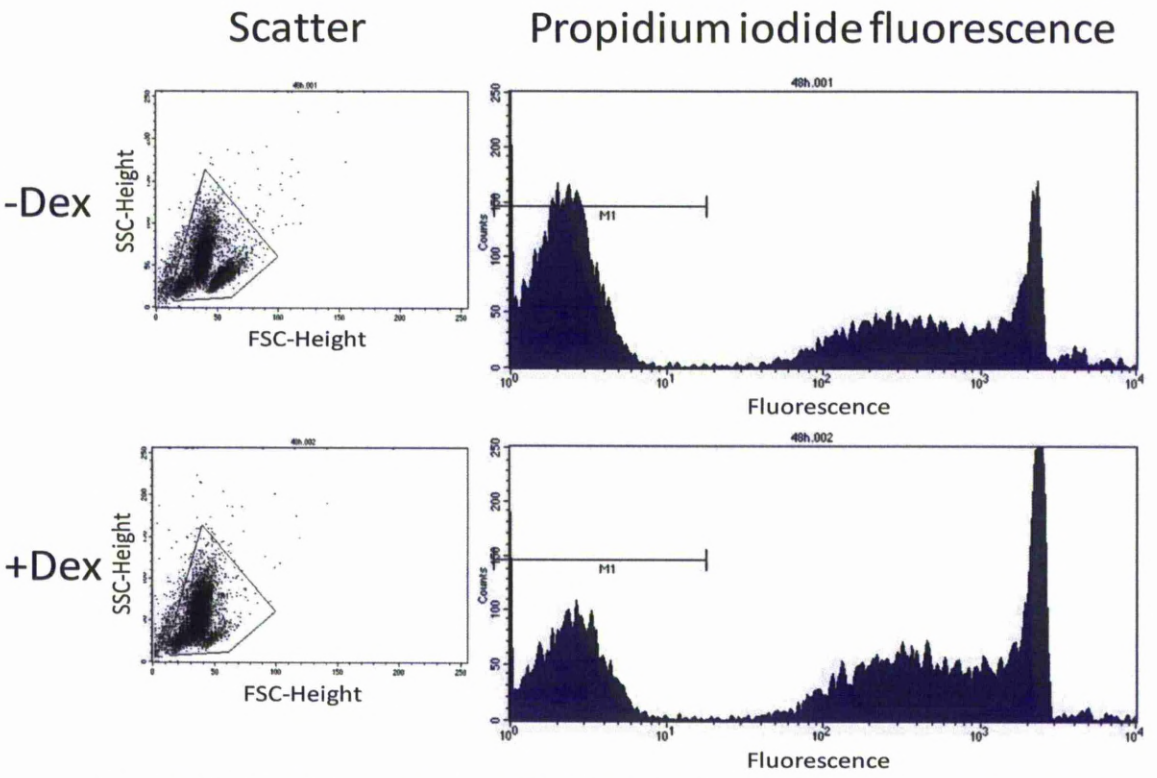


Figure 2.2.1. Representative FACS plots showing viability measurement by PI exclusion. Following 48h culture with/without (w/wo) 100nM Dex, CLL samples were stained with 2.5μg/ml PI and analysed by FACS. The scatter plots indicate cell size and granularity by forward and side scatter, respectively, allowing intact live and dead cells to be segregated from cellular debris (left of the gated region). The histograms show red fluorescence due to PI. The gate, M1, marks cells that are PI-dim, and thus represent live cells. The upper plot and histogram show CLL cell viability in an untreated control (-Dex) while the lower plot and histogram show viability in the same sample after *in vitro* exposure to 100nM Dex (+Dex).

2.2.6.2 DiOC6 (3,3′-dihexyloxacarbocyanine) and PI viability measurement

$DiOC_6$ (3,3′-dihexyloxacarbocyanine) is a green fluorescent molecule that is actively concentrated in the respiring mitochondria of live cells. Loss of $DiOC_6$ staining indicates disruption of the mitochondrial transmembrane potential

(Tedeschi 1974). This is an early and irreversible event during the induction of apoptosis (Brenner, Kroemer 2000). Therefore, apoptotic cells show decreased $DiOC_6$ staining. Double staining with $DiOC_6$ and PI allows distinct populations of cells to be identified, ranging from viable cells (DiOC6 bright, PI dim) to early apoptotic ($DiOC_6$ dim, PI dim), to late apoptotic/necrotic ($DiOC_6$ dim, PI bright).

Cells in culture media were incubated with 25nM $DiOC_6$ (Sigma, Poole, Dorset, UK) for 15mins in a 37°C incubator prior to incubation with PI, and FACS analysis as described in Section 2.2.6.1.

2.2.6.3 Annexin V and PI viability measurement

Annexin V binds to Phosphatidylserine, which is exposed on the surface of apoptotic cells and is thus considered an early marker of apoptosis induction (Koopman, Reutelingsperger et al. 1994). Double staining with PI allows the identification of live cells (annexin V dim, PI dim), early apoptotic cells (annexin V bright, PI dim) and late apoptotic/necrotic cells (annexin V bright, PI bright).

$4x10^5$ cells in volumes of 200µl were transferred to FACS tubes and washed in PBS. 400µl of binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$) was added to each sample and mixed by pipetting, before adding 3µl Annexin V FITC (BD Biosciences). Samples were incubated for 10min at R/T (room temperature) in the dark before incubation with PI and FACS analysis as described in Section 2.2.6.1.

2.2.7 Measurement of drug-induced cell killing by Tumour Response to Antineoplastic Compounds (TRAC) assay

2.2.7.1 Drug-coated plate make-up

Drugs were dissolved in appropriate solvent according to manufacturer's instructions, and serial dilutions were made up at a range of concentrations known to facilitate the elucidation of LC_{90} values. Concentrations were checked by UV spectrophotometry. Vacuum concentration for 40-80 minutes at medium heat was used to bind the drug to appropriate wells of the 96-well plates. Drugs were plated out at concentrations ranging from below to very much higher than concentrations considered therapeutically relevant.

2.2.7.2 Sample preparation

Samples from the university of Liverpool Biobank were removed from liquid nitrogen storage and transferred immediately to dry ice, on which they were transported by air to Royal United Hospital, Bath (for TRAC drug sensitivity screening). Upon receipt, the cells were transferred to a -80°C freezer. The cryopreserved cells (1ml) were thawed rapidly at 37°C and resuspended to a final volume of 8-12mls with Complete CO_2-independently buffered medium. Following centrifugation at 500rcf for 5 minutes, cells were washed in 1ml of Wash CO_2-independently buffered medium, supplemented with Gentamicin and L-Glutamine. Samples were then counted and resuspended in Ultracure Set-up medium (DMEM plus Bovine Extracts and Human Insulin), supplemented with

Gentamicin and L-glutamine. Cells were incubated at $3.3x10^6$ cells/ml, at 37°C and 5% CO_2, in tissue culture plates with U-shaped wells, to facilitate clustering, and an estimate of initial viability was made for quality control purposes.

2.2.7.3 Take off and staining

After 4 days, cell culture plates were removed from the incubator and cells were transferred by pipetting to Octospot slides held within holding blocks. The assembled blocks were briefly agitated, and the slides then spun on a cytospin at 1200rpm for 4 minutes. The slides were dried under a stream of cold air for a few minutes and then stained by Eosin/Thiazin using an Aerospray Slide Stainer.

2.2.7.4 Scoring of TRAC assay slides

Day 4 control slides were observed to estimate the percentage of malignant cells. If 20 live malignant cells could not be seen in the control spots, the assay was deemed invalid. Counts of representative spreads of cells were performed using 10 by 10 square eyepiece graticule inserted into the microscope eyepiece. When possible, approximately 100 cells were counted by selecting random fields. The average figure for the various control spots was taken to be "100% tumour survival". The pattern and density of cells per field were carefully considered, to be combined with the 100% tumour survival figure in estimation of drug treated slides. The drug treated slides were then scored by comparison to the untreated

control of the slide being studied and the control slide. The scores were predefined as 0,1,5,10,15,20,30,40,50,60,70,80,90, and 100% relative viability.

The LC_{90} position is defined as the lowest drug concentration at which 90% of malignant cells are killed; i.e. 10% are left alive as compared with control. For this reason, if 12-15% of cells were considered alive 15% was recorded. If no live cells could be seen a count of 0% was recorded, and 1 cell was noted as 1%. To ensure accuracy, all scores were performed twice by different operators (kindly facilitated by Biomedical Scientists at Bath Royal United Hospital Cancer Research). On the few occasions when pass thresholds for scoring discrepancies were breached, slides were repeat scored by both operators. LC_{90}s were calculated as previously described (Bosanquet, Bell 1996).

2.2.8 Preparation of protein samples and Western blotting

Cell samples were harvested and lysed in 200µl SDS Sample Buffer, containing 125mM Tris-HCl (pH6.8), 20% glycerol (Sigma), 4% SDS (Fisher Scientific, Loughborough, Leicestershire, UK), 10% β-mercaptoethanol (Sigma) and 0.006% bromophenol blue (VWR International, Lutterworth, Leicestershire, UK). The samples were sonicated at 30Hz in pulses 5secs in length, and then heated for 15mins at 95°C prior to being loaded for Western blotting.

Polyacrylamide gel electrophoresis (PAGE) was performed using running gels of 12-15% Ultrapure Protogel 37.5:1 Acrylamide:Bis-acrylamide (National Diagnostics), and stacking gels of 5%. 20µl of each CLL cell lysate was loaded. Proteins were separated by electrophoresis in Electrophoresis Buffer (0.025M

Trizma Base, 0.192M Glycine, 0.1% SDS) at 30mA/gel, and were then transferred onto Immobilon-P PVDF membranes (Millipore, Watford, UK) in transfer buffer (0.025M Trizma Base, 0.192M Glycine) at 400mA for 1h per transfer unit. Membranes were blocked for at least 30mins in 5% non-fat powdered milk (Tesco, UK) in TBS plus Tween-20 (TBS-T; Sigma), before incubation with primary and secondary antibody combinations as required. Wash steps consisted of 4 steps of 5mins each in TBS-T (0.1% Tween-20, Boros, UK).

Membranes were probed with primary antibodies in TBS-T for 1h at room temperature or overnight at 4°C as suggested in the manufacturers' instructions, followed by peroxidase-conjugated goat polyclonal anti-rabbit or anti-mouse secondary layer antibody (Santa Cruz, CA, USA), as specified in Table 2.2.1. Proteins were visualised by enhanced chemiluminescence using Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, US), or Amersham ECL Advance Western blotting Detection Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), as instructed by the manufacturers.

Table 2.2.1 Antibodies used in immunoblotting. Antibody incubations were for 1h at R/T or O/N at 4°C.

Primary Antibody	Dilution	Secondary Antibody	Dilution
PARP Mouse PAb RnD Systems	1/4000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
β-Actin Mouse MAb AC-74 Sigma	1/10000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000

2.2.9 Clinical data

All CLL patient samples used in this study were obtained with informed consent for storage in the University of Liverpool Leukaemia Bank and use in a wide range of scientific studies. Clinical data were extracted from patient's medical notes and stored in a password protected central departmental database (in accordance with data protection regulations).

2.3 Results

2.3.1 Determination of the optimal Dex dose and incubation period

The primary aim of this investigation was to establish that CLL cells show varying degrees of sensitivity to Dex *in vitro*, to allow selection of the most sensitive and resistant samples for comparison studies. It was first necessary to determine appropriate concentrations of the compound for screening of a representative cohort of patient samples. Killing was measured by PI exclusion and FACS (Section 2.2.6.1). Figure 2.3.1 displays the effect of a range of Dex concentrations on 4 CLL samples over the course of 3 days. Most of the reduction in viability of sample 2499—the most sensitive case—was observed at a Dex concentration of between 1 and 10nM Dex, and killing began to plateau as the Dex concentration reached 100nM. In all samples viability was very similar at Dex concentrations between 100nM and 1000nM, though higher doses did appear to achieve slightly more killing in at each timepoint. Killing was seen as early as 24h, whilst variation in sensitivity between samples was more apparent by 48h. 100nM Dex was the lowest dose to induce close to maximum killing over the course of 3 days, thus highlighting this dose as potentially useful in further studies. The low standard deviations observed between triplicate samples provided confidence that this assay was of sufficient accuracy.

2.3.2 Establishing a CLL Dex-response profile

A panel of CLL samples were initially screened for their sensitivity to killing by 100μM Dex (n=51). Available samples (n=46) from 44 patients (Table 2.3.1) were also tested for their sensitivity to the lower dose of 100nM Dex in order to deduce whether sensitivity was consistent at a more clinically relevant dose.

Figure 2.3.2 shows samples ranked by Dex sensitivity at 48h. Killing data at this timepoint gave a clear separation of samples without compromising the assay with the high spontaneous death that was apparent in certain samples by 72h. The most distal samples in the sensitivity spectrum were then arbitrarily assigned as sensitive and resistant. It is clear that Dex sensitivity is spread evenly from the most sensitive to the most resistant case. Of particular note, sensitivity at 24h does not correlate well with sensitivity at 48h or 72h, whilst 48h and 72h killing values coincide much more closely. The lack of a correlation between 24h and 48h killing possibly reflects a killing mechanism that features multiple pathways that are activated at differing rates.

Table 2.3.1 Clinical and laboratory variables for 44 CLL patients/46 CLL samples (pairs of samples from separate bleeds of 2 patients were included)

Sex (patients)	**Male: 25** **Female: 19**
Age at diagnosis (patients)	**Mean: 67.2 (95% CI: 64.2-70.2)**
Prior therapy (patients)	**Yes: 20** **No: 24**
WBC (10^9/L) (samples)	**110.5 (95% CI:89.5-131.5)**
***IGHV* (samples)**	**Mutated: 19/42** **Unmutated 23/42**
Cytogenetics (samples)	**17p-: 5/27** **11q-: 2/26** **+12: 5/23** **13q: 22/32**
p53 dysfunction (samples)	**Normal : 20/35** **Abnormal:15/35**

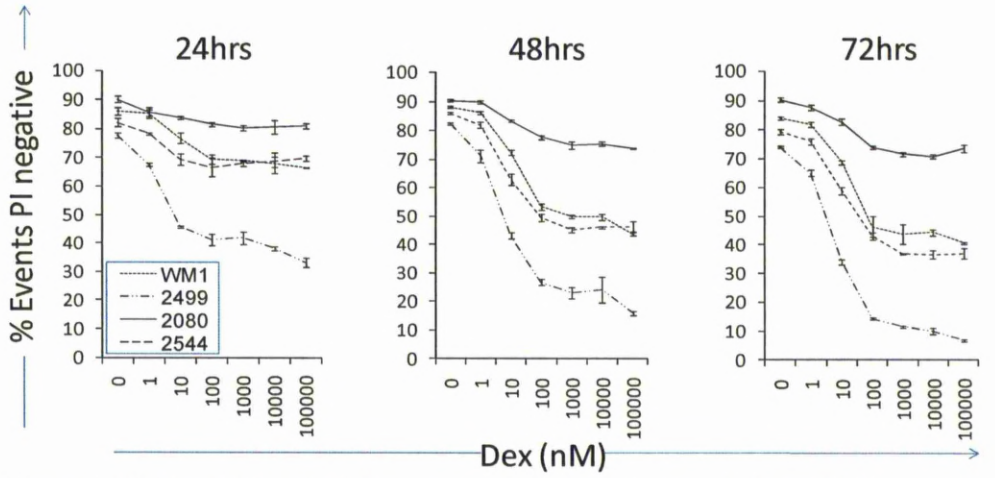


Figure 2.3.1. Dex dose/response curves for one GC-sensitive, one GC-resistant, and two GC-intermediate-response CLL samples. CLL samples were treated with a range of doses (in triplicate) of Dex, ranging from between 0 and 100000nM Dex for 24, 48, and 72h. The fraction of cellular events collected that were alive—"PI Dim"— is shown, as determined by FACS. Each line represents a separate case. The error bars represent the standard deviation of 3 samples.

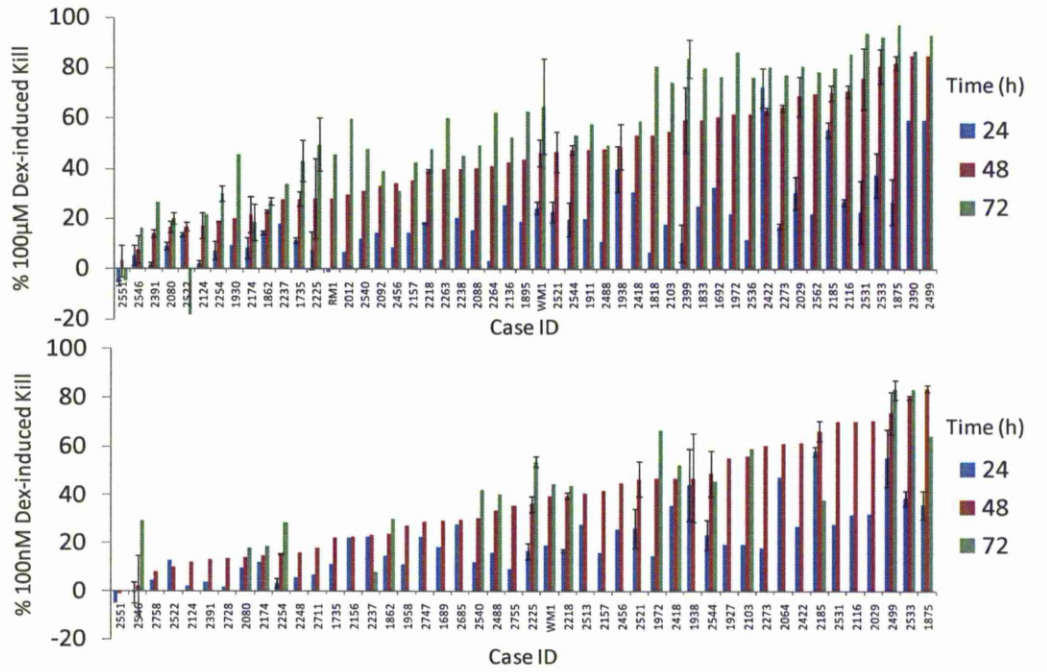


Figure 2.3.2. Dex sensitivity spectrum for a panel of CLL samples. Top. A panel of 51 CLL samples was screened at up to 3 timepoints to determine response to a saturating dose of Dex (100µM), as measured by PI exclusion/FACS. Bottom. 46 samples were also tested with a lower dose of 100nM Dex. The bars represent relative killing relative to untreated controls as determined by PI fluorescence. Standard deviation for one separate independent experiment is shown.

The sample/patient cohort used to designate samples for further study is shown in Table 2.3.1. This cohort is fairly representative according to epidemiological data. More specifically, more male than female patients were included, which is consistent with observations that CLL is more common in males than females (Gribben 2010); and mean age at diagnosis was 67.2yrs, which is close to the 72yrs average reported by Dores *et al* (Dores, Anderson et al. 2007) (Section 1.1.2). A minority of samples were from patients with deletions of 17p or 11q, or with trisomy 12, which is consistent with reports suggesting that these three aberrations occur in less than 25% of patients (Zenz, Mertens et al. 2010, Oscier, Matutes et al. 1997) (Section 1.1.3). ~50% of patients had received prior therapy, whilst *IGHV* was mutated in ~50% of samples. p53 dysfunction was present in approximately half of the samples for which data was available.

100nM and 100μM killing scores were directly compared (Figure 2.3.3) (24h n=33, R^2=0.688, ***P***<0.005; 48h n=33, R^2=0.947 ***P***<0.005; 72h n=19, R^2=0.755, ***P***<0.005). The strong correlations observed for killing at 1, 2, and 3 days support the use of the 100nM data for determination of GC resistance and use in future experiments, and also support the idea that GC resistance in the sample cohort is not simply a dose effect that can be easily overcome.

Calcium release from the mitochondria of CLL cells has been detected as early as 6h, and caspase activity as early as 3h in response to GC treatment (Chandra, Gilbreath et al. 1997, McConkey, Chandra 1999). Thus, to further define the kinetics of Dex-induced killing in the samples of this study, killing was measured between 2h and 20h in 3 sensitive samples (Figure 2.3.4). Cells were stained with

$DiOC_6$ in addition to PI so that early as well as late apoptotic events could be quantified (Section 2.2.6.2). It is noteworthy that 2/3 samples showed clear evidence of Dex-induced killing from as early as 12h. Case 2116 shows more prominent spontaneous death than samples 2273 and 2533, illustrating the heterogeneity of these clinical samples. It is also clear that killing was less marked when only PI was used to measure viability, as would be expected.

To evaluate the possible impact of variation in cell density between experiments, GC sensitivity was compared using CLL cells seeded at different densities. Thus, CLL cells from two sensitive samples (2273, 2533) and one resistant (2546) sample were seeded at $2x10^6$/ml and $5x10^6$/ml and exposed to 100nM Dex for 24h and 48h. No significant difference in Dex-induced killing was observed between CLL cells seeded at the two cell densities ($P>0.05$) (Figure 2.3.5). At 48h, the maximum change in sensitivity was less than 10%. More importantly, this change did not alter the sensitivity grouping of the samples.

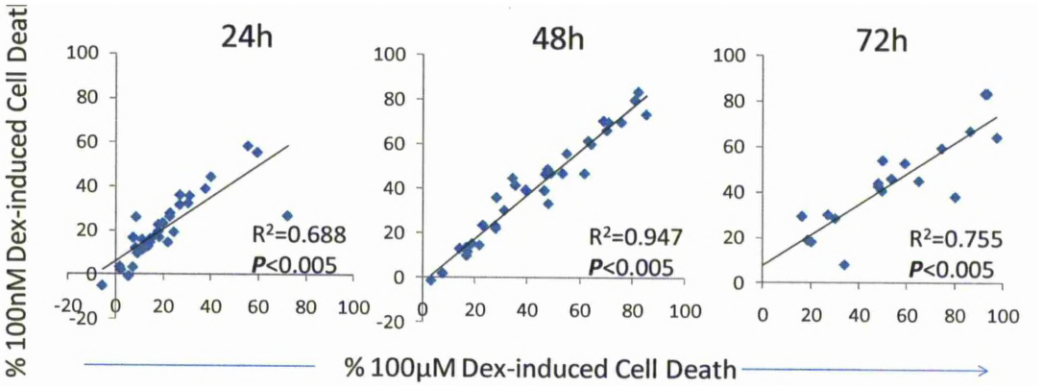


Figure 2.3.3. 100nM and 100μM Dex sensitivity data strongly correlate. The separate sets of sensitivity data shown in Figure 2.3.2 were compared using linear regression: 24h n=33, R^2=0.688, ***P***<0.005; 48h n=33, R^2=0.947 ***P***<0.005; 72h n=19, R^2=0.755, ***P***<0.005.

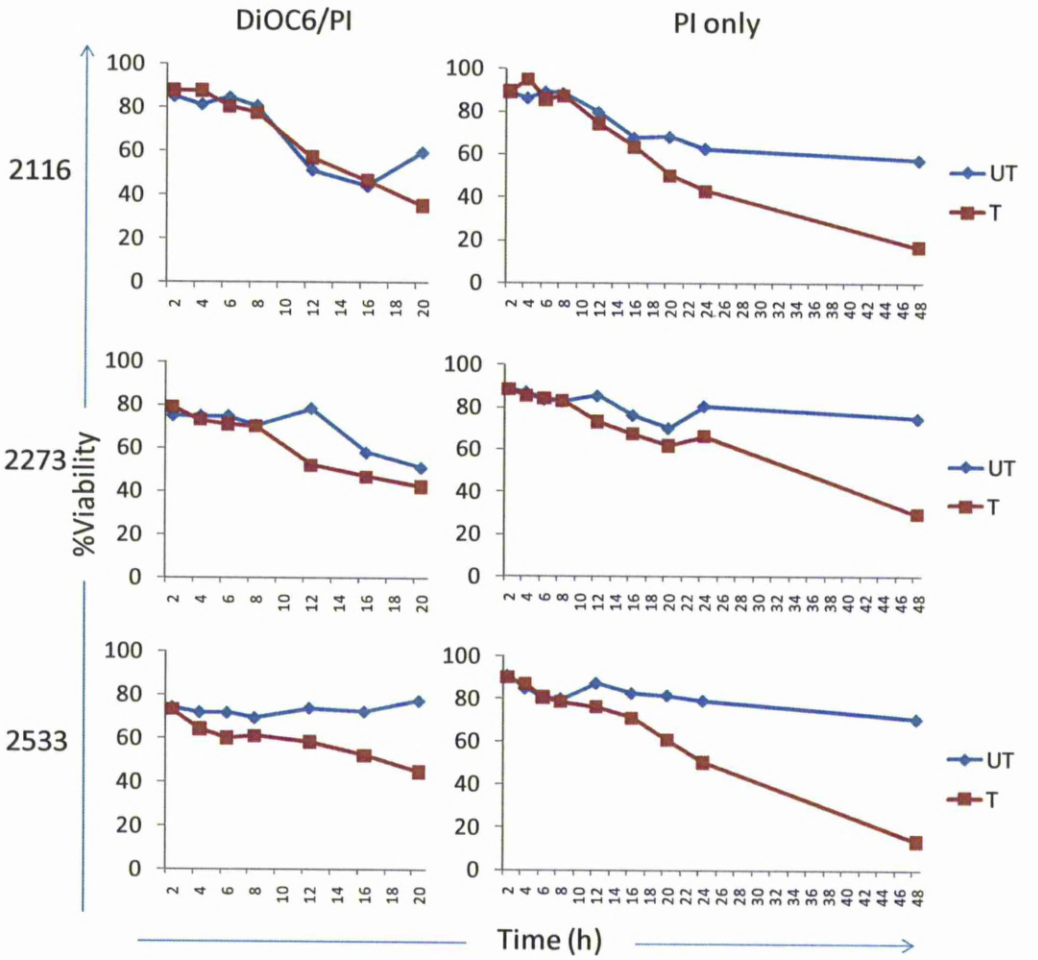


Figure 2.3.4. Dex begins to kill sensitive samples as early as 4 hours after treatment. Samples were cultured for 2, 4, 6, 8, 12, 16, and 20h and viability was detected by DiOC6/PI staining and FACS analysis. Percentage viability for the $DiOC_6$/PI charts (left) represent the proportion of cells that are $DiOC_6$+/PI-, whilst the PI only charts represent the proportion of cells that are PI- only (right).

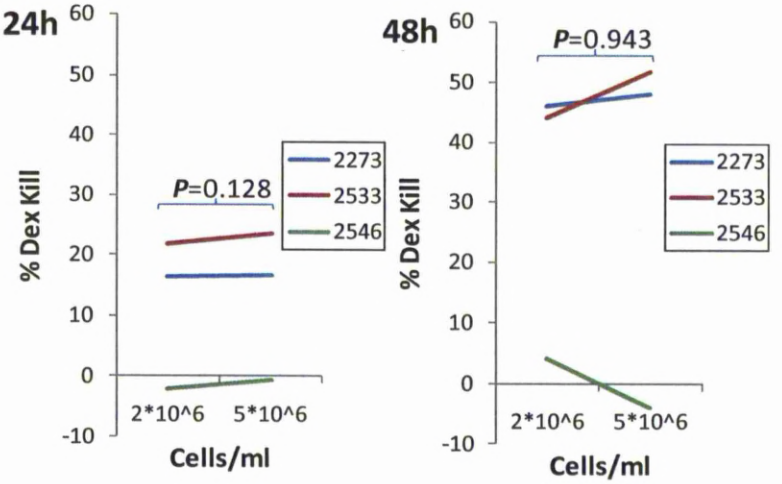


Figure 2.3.5. Cell concentration/density does not significantly alter Dex sensitivity as measured by Propidium Iodide/FACS. 3 CLL samples were cultured at two cell concentrations/densities for 24 and 48h, and the effect of Dex on killing was contrasted by PI/FACS. ***P***-values represent 2-tailed *t*-tests for difference.

2.3.3 Validation of Dex-induced CLL cell death measured by PI/FACS with TRAC (Tumour Response to Antineoplastic Compounds) assay

To further increase confidence that the samples for further examination were representative of sensitive and resistant phenotypes, a selection of samples were screened using the established TRAC (Tumour Response to Antineoplastic Compounds) assay, as described in Section 2.2.7. Whereas the PI/FACS survival assay featured only one or two doses of Dex (Section 2.3.2), this 4-day chemoresponse assay provided LC_{90} values, as samples were treated with a range of concentrations of the cytotoxic agents used. LC_{90} is defined as the concentration of an agent that is required to kill 90% of cells in a sample. Therefore, it provides a more robust measurement of drug sensitivity than simply measuring the killing at a fixed dose. The TRAC assay also conserves patient material by allowing use of a relatively low number of cells, as compared to other commonly used killing assays such as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Another advantage of this method is that cells can be identified morphologically at the end-point, thus avoiding issues with sample purity. There is compelling data to suggest that choice of chemotherapy in CLL can be effectively guided by TRAC assay (Bosanquet, McCann et al. 1995).

As Figure 2.3.6 shows, there was a clear correlation between the measurements of these two assays, thus supporting the concept of using the PI method to define sensitive and resistant samples ($n=34$, $R^2=0.579$, $P<0.005$).

2.3.4 Comparison of *in vitro* sensitivity of CLL samples to Dex with sensitivity to fludarabine and other GCs

It is well established that the nucleoside analogue fludarabine kills CLL cells through p53-dependent mechanisms (Pettitt, Sherrington et al. 1999, Pettitt 2003), whereas GC killing of CLL cells is not dependent upon the integrity of the p53 pathway (Thornton, Matutes et al. 2003). By comparing the effect of these different agents on CLL cell viability it was possible to deduce whether the GC resistance mechanism was one of general drug resistance (Figure 2.3.7). Furthermore, by screening the other GCs 6-Methylprednisolone (Mep) and Hydrocortisone (Hz) it was possible to define GC resistance of the CLL sample cohort as either specific or broad-acting. Figure 2.3.7 (B, C, and D) shows that the correlations as measured by linear regression between sensitivity to different GCs are significant, with all ***P***-values below 0.05 and R^2 above 0.7. In keeping with a previous study in CLL (Bosanquet, McCann et al. 1995) fludarabine sensitivity did not correlate with Dex sensitivity. Thus, GC resistance in the samples of this study was not likely to be a multi-drug resistance mechanism, and GC sensitivity within this investigation could be further defined as to apply to GCs in general, and not Dex in isolation.

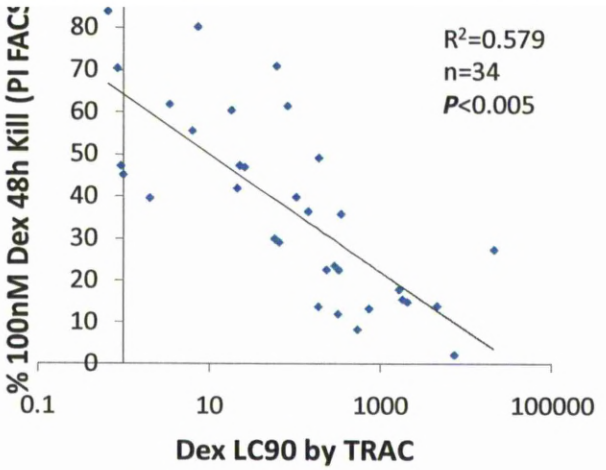


Figure 2.3.6. Dex LC_{90} as derived by TRAC assay correlates with PI/FACS assay killing data. LC_{90}s were derived from a panel of up to 38 samples using TRAC assay scores following 4 day culture, and compared with 48h killing data from Figure 2.3.2. LC_{90}s are shown on a logarithmic scale. Linear regression was performed: n=34, R^2=0.579, ***P***<0.005.

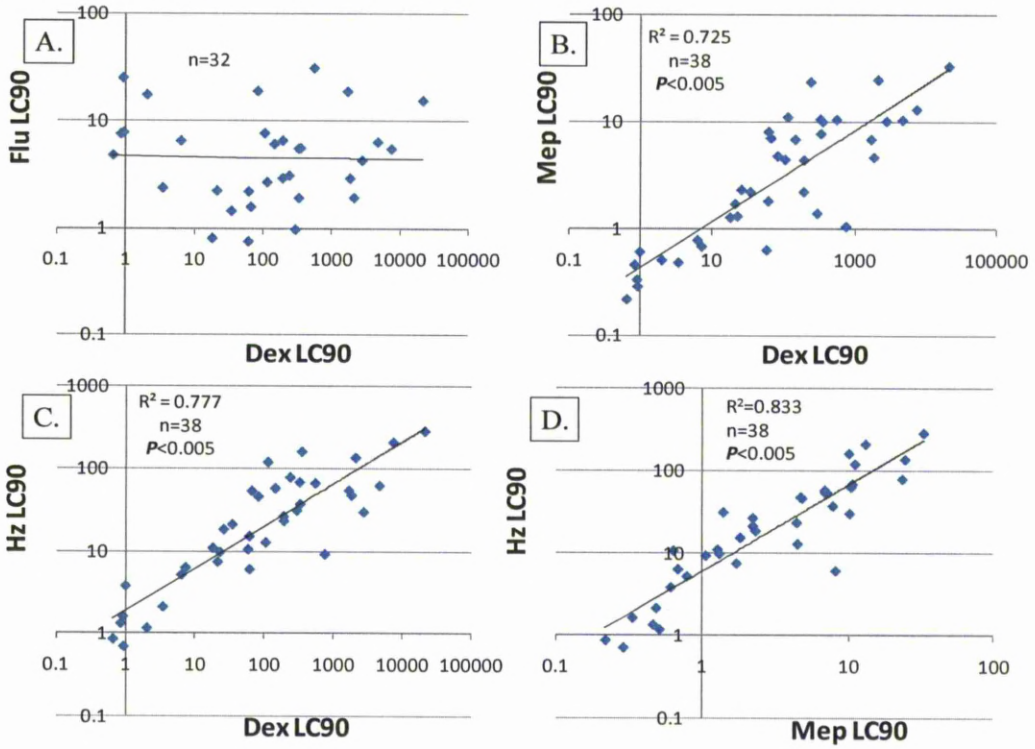


Figure 2.3.7. Dex sensitivity does not correlate with fludarabine sensivity, though GC sensitivities all strongly correlate. Fludarabine sensitivity does not correlate with Dex sensitivity (A), whereas GC sensitivity is strongly correlated (B/C/D). LC_{90}s were derived from a panel of up to 38 samples, using TRAC assay scores following 4 day culture. Plots of LC_{90}s are shown on logarithmic scales. Power regression was performed for: A. Dex v Flu (n=32); B. Dex v Mep (6-methylprednisolone) (n=38, R^2=0.725, ***P***=<0.005) ; C. Dex v Hz (hydrocortisone) (n=38, R^2=0.777, p<0.005); D. Mep v Hz (n=38, R^2=0.833, p<0.005).

2.3.5 Apoptosis contributes to Dex-induced CLL cell death

Indicators of apoptosis have previously been observed in CLL cells in response to Dex treatment (Tiwari, Dong et al. 2005, Chandra, Gilbreath et al. 1997, McConkey, Chandra 1999). It was decided to confirm the occurrence of apoptosis in the samples of this study. Phosphatidylserine (PS) exposure is a marker of apoptosis as it is exposed on the surface of apoptotic cells from early through late apoptosis (Koopman, Reutelingsperger et al. 1994) (Section 2.2.6.2). FITC-conjugated annexin-V binds to PS, and is detectable by FACS. A group of sensitive and resistant samples were tested for annexin-V staining and PI exclusion concomitantly (Figure 2.3.8). Samples were cultured for 24h in order to facilitate a commitment to killing without causing sensitive samples to completely enter the late apoptotic phase (Figure 2.3.4). PI-bright/annexin-bright events (upper right) represent late apoptotic or necrotic cells, whilst the PI-dim/annexin-bright events are early apoptotic (upper left). The detection of the latter population indicates that death was occurring by apoptosis as opposed to necrosis. In confirmation of the sensitivity designations, the sensitive samples showed viability reduction of between 32% and 58%, whilst the resistant samples displayed only 2% and 16% reductions in viability.

A further marker of apoptosis is Poly-ADP Ribose Polymerase (PARP) cleavage, as PARP is cleaved by effector caspases as a downstream event in apoptosis in CLL cells (Pettitt, Cawley 2000). Figure 2.3.9 shows by Western blotting that PARP was indeed cleaved in response to Dex in CLL samples, and also shows a higher propensity for this phenomenon in sensitive than resistant samples, indicating that apoptosis was more prevalent in the most sensitive samples.

Sample 2185 of the sensitive group appeared to show the greatest PARP cleavage. It is of note that the ratio of cleaved to uncleaved PARP is higher at 12h than 24h, which possibly indicates that cleaved PARP is degraded more rapidly than it is formed after 24 hours in these samples. As is the case with both killing (Figure 2.3.2) and PS exposure (Figure 2.3.5), resistant samples did show some PARP cleavage, rather than a complete absence. However, overall the sensitive samples showed considerably more Dex-induced PARP cleavage at both time points. Taken together, the PS exposure (Figure 2.3.8) and PARP cleavage data supports a role for apoptosis in the GC-induced killing of CLL cells, which is in keeping with previous studies.

In order to confirm the idea that caspase activation was not an essential component of killing, as has been reported previously (Kroemer, Martin 2005), two sensitive samples were cultured for 24 and 48h w/wo 100nM Dex and the caspase inhibitor z-vad.fmk. Z-vad.fmk appeared to protect only 1/2 samples (hence all $P>0.05$) from loss of mitochondrial integrity as measured by loss of $DiOC_6$ positivity (Figure 2.3.10). However, this effect was of a lower order of magnitude when PI positivity rather than $DiOC_6$ negativity was used to measure killing. Therefore, caspase activation is likely to contribute to Dex-induced killing of certain CLL samples, though it does not appear to be an essential component (Figure 2.3.9).

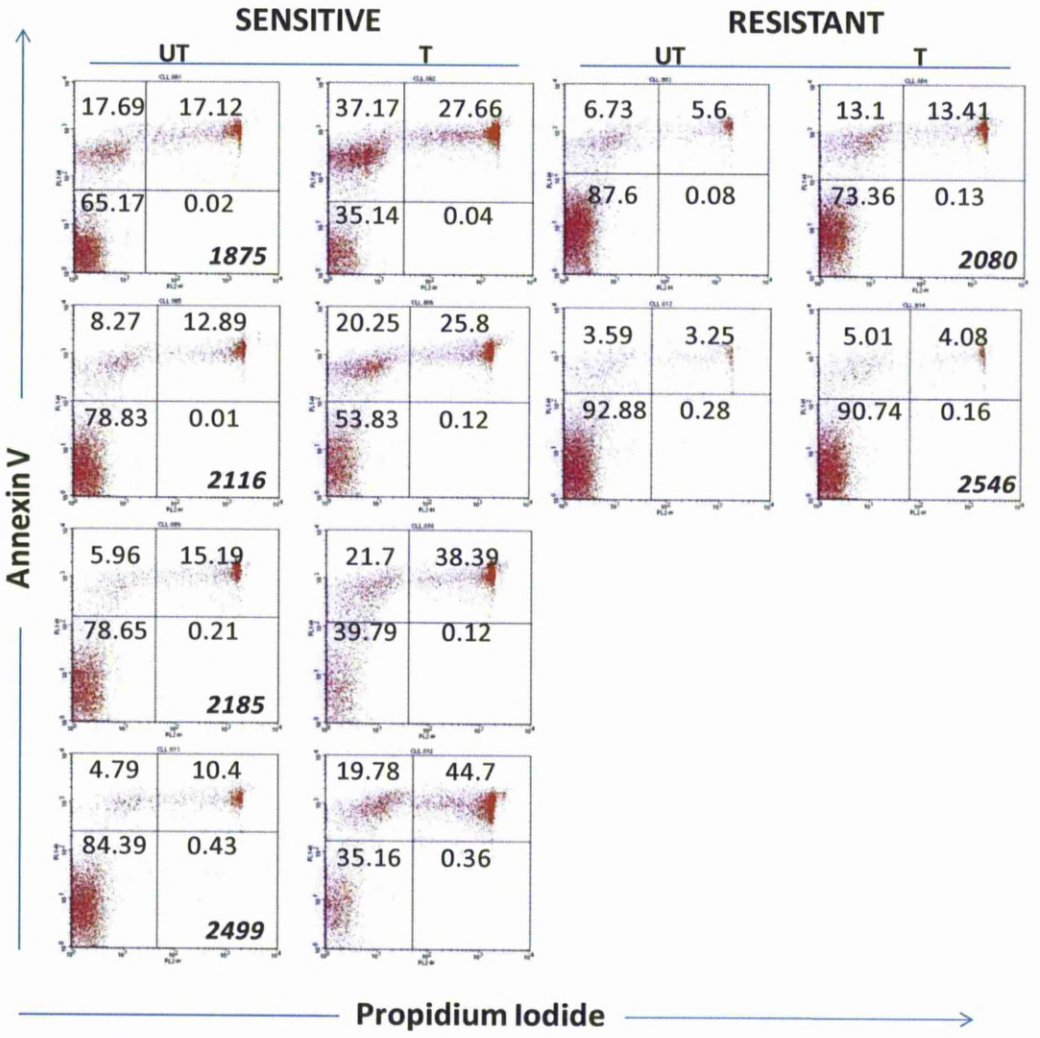


Figure 2.3.8. Dex induces cell killing via a mechanism that involves phosphatidylserine exposure. 4 sensitive and 2 resistant samples were cultured for 24h w/wo 100nM Dex. Cells that appear to be annexin V and PI null are considered viable (lower left). Annexin V positivity alone indicates early apoptosis (upper left), whilst dual positivity indicates late apoptosis (upper right).

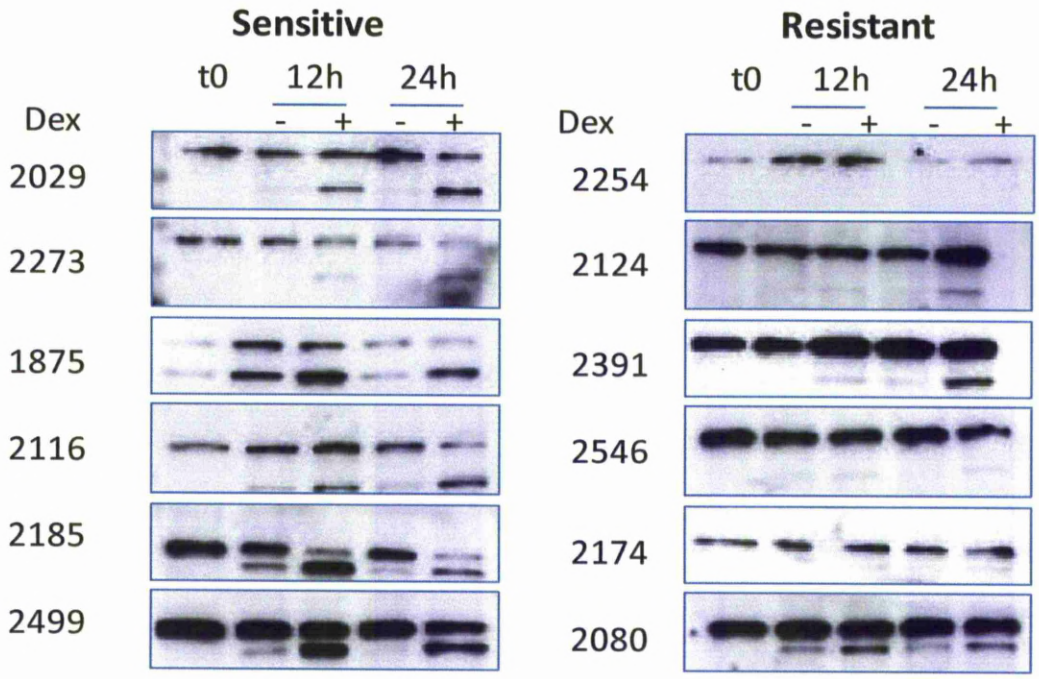


Figure 2.3.9. PARP cleavage is more readily detectable in sensitive than resistant samples. Western blots showing uncleaved (113kDa) and cleaved (89kDa) PARP CLL samples were cultured for 0h, 12h, and 24h w/wo 100nM Dex. Lysates were produced using SDS-sample buffer, and the resulting samples were separated by SDS-PAGE.

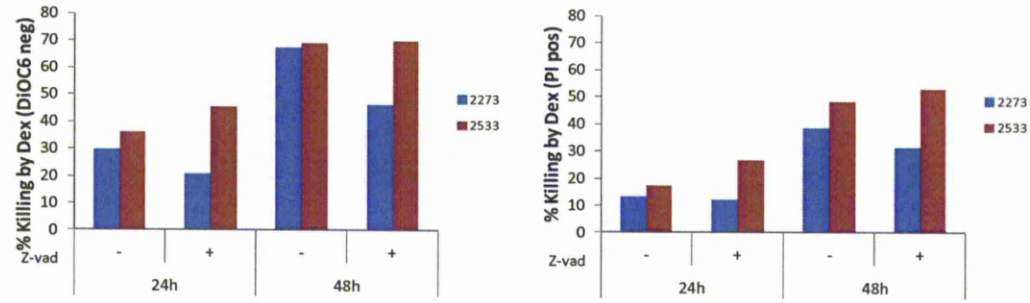


Figure 2.3.10. Caspase inhibitor does not rescue sensitive samples from the killing effect of Dex. Two sensitive cases were cultured for up to 48h +/- 100nM Dex in the presence or absence of z-vad.fmk. Left. Killing as measured by mitochondrial membrane depolarisation (24h ***P***=0.99, 48h ***P***=0.52 paired t tests for difference). Right. Killing as measured by PI staining (24h ***P***=0.57, 48h ***P***=0.89 paired *t* tests for difference).

2.3.6 Clinical and laboratory variables for study samples

It was important to compare clinical and laboratory variables to Dex sensitivity scores in case mechanistically relevant variables were highlighted, and to further confirm the validity of the sample cohort. *IGHV* mutation status is an important predictor of poor prognosis (Hamblin, Davis et al. 1999). Samples with UM-*IGHV* (n=23) had a median sensitivity approximately two-fold higher than those with M-*IGHV* (n=19) (Figure 2.3.11). As unmutated *IGHV* predicts a more aggressive form of the disease (Lin, Sherrington et al. 2002), this correlation illustrates the potential usefulness of Dex in poor prognosis patients, and is in keeping with a previous study of GC sensitivity in CLL (Aleskog, Tobin et al. 2004).

Table 2.3.2 displays selected clinical and laboratory variables for each of the samples designated either GC sensitive or GC resistant. When the two sensitivity groups are contrasted (Table 2.3.3) it appears that mean age at diagnosis, mean white blood cell count (WBC), and Binet stage distribution were similar. However, there was a higher ratio of females to males in the resistant group (resistant=1, sensitive=0.33). Moreover, there was a much higher ratio of M-*IGHV* to UM-*IGHV* in the resistant group (resistant=2.5, sensitive=0.22), in keeping with Figure 2.3.11. Ratio of p53 dysfunction to "normal" was higher amongst resistant than sensitive cases (resistant=0.75, sensitive=0.375), though the majority of cases within both groups are "normal". Furthermore, there was a higher ratio of previously treated to untreated patients in the sensitive group (sensitive=1, resistant=0.45), which supports the reported effectiveness of GCs in treating refractory CLL (Thornton, Hamblin et al. 1999).

Previous treatment of patients with chlorambucil can sensitise CLL cells to GC-induced killing (Bosanquet, McCann et al. 1995) (Section 1.4.5), though in this study 4/16 resistant as well as 4/12 sensitive patients had previously been treated with chlorambucil.

Regarding FISH data, *ATM* deletion (11q) was rare in both groups (ratios to "normal"=0.111), as was 11q/14q translocation (1/16 cases). Most cases displayed a deletion of 13q, which was more prevalent in the sensitive cases (ratios to "normal"; sensitive=2.33, resistant=1.4). This was unsurprising given that these deletions occur in around 50% of patients (Fitchett, Griffiths et al. 1987). Whilst trisomy 12 was rare, it was more common in resistant cases (ratios to "normal"; sensitive=0.111, resistant=0.5).

TP53 deletion/mutation is the most clear indicator of poor treatment outcome in CLL (Furman 2010). In keeping with the p53 dysfunction data, *TP53* deletions were more common in the resistant group (ratios to "normal"; resistant=0.57, sensitive=0.111). This finding is in contrast with previous reports that suggest a redundant role for p53 in GC-induced killing of CLL cells (Pettitt, Matutes et al. 2006, Thornton, Matutes et al. 2003, Clarke, Purdie et al. 1993).

If spontaneous death *in vitro* were linked to Dex sensitivity, defects in CLL survival would be implicated in the phenomenon of Dex resistance. Figure 2.3.12 shows the lack of a statistically significant correlation between these two variables, and so it is not likely that defects in generic CLL survival signalling are responsible for Dex resistance.

Table 2.3.2 Clinical and laboratory variables for GC sensitive and GC resistant samples

[*=age at first sample, **=estimate based upon LC, (P)=data from previous bleed, (S)=data from subsequent bleed].

Sample Group	100nM Dex 48h % kill	Sample ID	Age at diagnosis (years)	Sex (M/F)	WBC (10^9/L)	LC (10^9/L)	Binet	Previous treatment	VH%	Class	p53	17p-	11q-	13q-mono	13q-bi	Trisomy 12	11q14q translocation	bleed to FISH (months)
Resistant	-1.235741445	2551	54*	F	52.4	46.2		CLB/FLU	1.75 (P)	M	B	16/200	16/200	Normal	Normal	83/100	Normal	2
	2.049048382	2546	52	M	69.0**	64.1		CLB	8.07 (P)	M	WT (P)							
	8.168028005	2758	74	M	60.2	57		Yes	0 (P)	M	B (P)	145/150	Normal	Normal	Normal	Normal	Normal	-24
	10.13303769	2522	73	F	86.4	79.3		No	5.21 (P)	M	WT P/S)	Normal	Normal	135/150		Normal	Normal	-19
	11.89307851	2124	62	M	50.3	42.7	A (P/S)	No	4.56	M	C	Normal	Normal		116/200		Normal	0
	13.17035905	2391	65	M	77.7	67.7	A (P/S)	No	6.25 (P)	M	B (P)	21/100	Normal	92/100		Normal	Normal	-22
	13.66482505	2728	65*	M	163	155		No	0 (P)	M		Normal	Normal	Normal	Normal	137/150	Normal	-21
	13.82743363	2080	69	F	119.1		A	No	8.93	M	B	Normal	Normal	Normal	Normal	Normal	Disrupted	0
	14.76846058	2174	40	F	102.9	93.4	A (P/S)	No	5.84 (P)	M	WT (P/S)	Normal	Normal	174/200	12/200	Normal	Normal	0
	15.3764525	2254	71	F	125	112	A	No	7.56	M	WT (P)							
	16.02108037	2248	75*	M	43.3	36.3	B	No	9.47	M	C							
	17.77941719	2711	71*	F	242.3			No	12.28	G		53/100			127/147			0
	22.38547969	1735	79*	F	39.8		A	No			WT							
	22.49022164	2156	61	F	183			CLB/PRED	0.34 (P)	M	WT	Normal	Normal	Normal	Normal	8/100	Normal	-2
	23.39449541	2237	41	M	210	203	B	No	8.59	M	WT			128/150				0
	23.75	1862	61	M	172			CLB/DEX			WT	Normal	Normal	85/100		Normal		0
Sensitive	55.45973014	1927	60	M	181			CLB/PRED	0.35 (P)	M	WT (P)	Normal	Normal	20/200		Normal		-1
	56.11192931	2103	55	M	125	119		No	0 (P/S)	M	A	100/105	Normal	67/241	9/241	Normal	Normal	0
	60.36601656	2273	54	M	51	45	A	Yes	0.27 (S)	M	WT	Normal	Normal	41/100		Normal	Normal	7
	61.34569173	2064	45	M	120			CLB	0.33	G	B	Normal	Normal	Normal	Normal	160/200	Normal	0
	61.7681597	2422	60*	F	98	86		No	0	M	WT							
	66.57927252	2185	63*	M	50.8			No			WT	Normal	Normal	Normal	Normal	Normal	Normal	0
	70.24015068	2531	61	M	47.4	41.6		No	0.35 (P)	M	WT	Normal	Normal	145/155	Normal	Normal	Normal	0
	70.30134123	2116	55	M	79.7	73.8		CLB/FLU	0 (P)	M	B	Normal	192/200	94/100		Normal	Normal	0
	70.83153192	2029	62	F	57.1			No	2.8	M	WT	Normal	Normal	50/100		Normal	Normal	-57
	73.78201545	2499	64	M	51.5	48.3	B	Yes	5.58	G	WT							
	80.17083029	2533	54	M	116.1	103		No	0	M	WT	Normal	Normal	150/158	Normal	Normal	Normal	0
	83.86372448	1875	73	F	310		C	CLB	0 (S)	M		Normal	Normal	Normal	Normal	Normal		-22

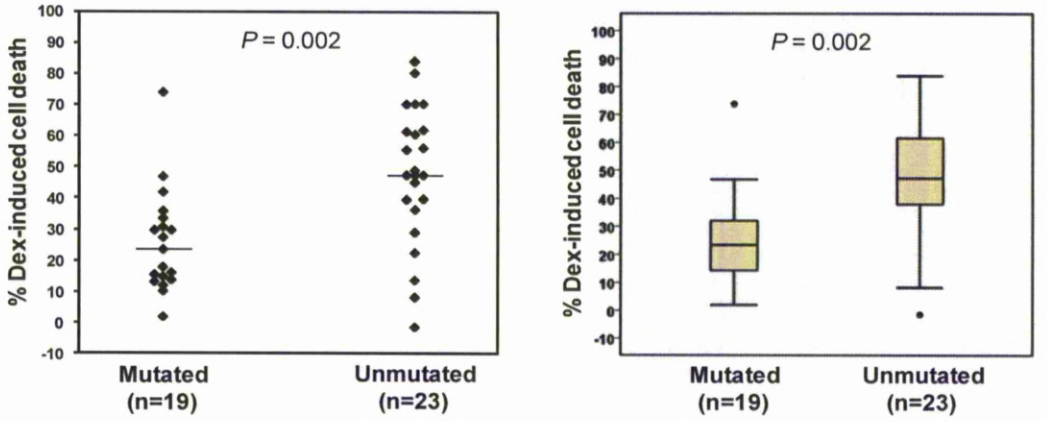


Figure 2.3.11. Samples with UM-*IGHV* tend to be killed more effectively by Dex than those with mutated versions. 48h 100nM Dex-killing data was compared to *IGHV* status in samples from the study cohort (n=8S/10R, Mann-Whitney *U* test ***P***=0.041). Mutation status was defined as <2%=unmutated.

Table 2.3.3 Clinical and laboratory variables by sensitivity group

	Sensitive	Resistant
Mean Age at Diagnosis (years)	63.3	58.8
Sex	9M/3F	8M/8F
Mean WBC	107.3	112.3
Binet (A/B/C)	1A/1B/1C	6A/2B
Previous treatment (Y/N)	6Y/6N	5Y/11N
IGHV (UM/M)	9UM/2M	4UM/10M
p53 dysfucntion (wt/dys)	8wt/3dys	8wt/6dys
17p- (del/norm)	1 del/9 norm	4 del/7 norm
11q- (del/norm)	1 del/9 norm	1 del/9 norm
13q- (del/norm)	7 del/3 norm	7 del/5 norm
Trisomy 12 (tri/norm)	1 tri/9 norm	3 tri/6 norm
11q 14q translocation (trans/norm)	0 trans/8 norm	1 trans/8 norm

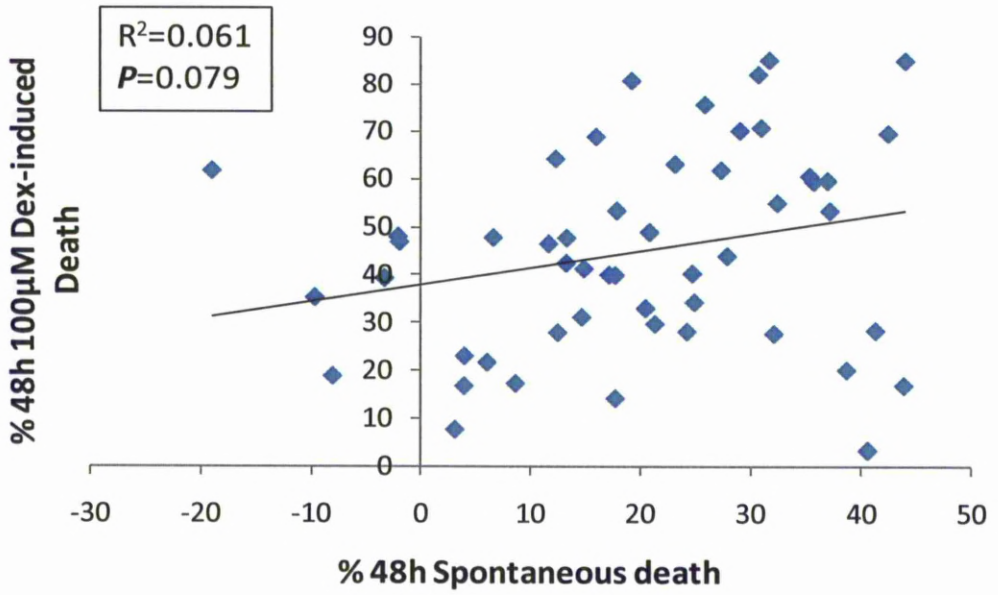


Figure 2.3.12 Spontaneous death of CLL cells during *in vitro* culture does not significantly correlate with Dex-induced killing. Spontaneous death of CLL samples was established by comparison of viability at t0 with viability at 48h (n=51, R^2=0.061, ***P***=0.079).

2.4 Discussion

The aims of this chapter were to define the sensitivity to Dex of a panel of CLL samples, and to characterise the mode of killing. Also, sensitivity to Dex-induced killing was related to clinical and laboratory variables.

In vitro GC-sensitivity assays narrow the search for GC resistance in CLL to the cellular context. This means that potential resistance mechanisms lying in pharmacokinetics, including sequestration of GC by plasma-borne proteins, and differing degrees of liver metabolism and excretion between individual patients cannot be considered. Also, the increasingly well defined involvement of *in vivo* microenvironment (Ghia, Circosta et al. 2005) (Section 1.2.3) was not tested as part of this investigation. Nevertheless, *in vitro* sensitivity can be considered clinically relevant as patient treatment response has been previously shown to correlate with the *in vitro* GC sensitivity of CLL samples (Bosanquet, McCann et al. 1995).

Changes in cell viability over the course of 3 days following treatment with Dex were primarily measured using an established PI flow cytometry method (Pettitt, Sherrington et al. 1999) that allowed detection of both apoptotic and necrotic cells. CLL cell samples showed a range of sensitivities to Dex treatment using this method. PI exclusion does not define mode of killing but it is a marker of cells that are in the latter stages of death, as fluorescence is seen to increase following perturbation of the nuclear membrane and binding of PI to DNA (Darzynkiewicz, Bruno et al. 1992). However, though this method involves gating on live and dead cells, it does not take into consideration cell count post-

culture, and so excludes obliterated cells and cellular fragments. This is likely to lead to an underestimation of the number of dead cells in a sample, and thus reduce the apparent range of GC sensitivity in the study cohort. However, sensitivity ranking should not be affected, and so the benefits of this high-throughput, rapid viability measurement outweigh the weaknesses. Previous experiments within this research group showed that GCs kill CLL cells gradually in culture over the course of days rather than hours, and so initial experiments were performed over a 3 day timecourse.

To further strengthen the data collected by flow cytometry, and to identify whether GC sensitivity correlated with that of fludarabine, a separate method was used on a panel of the samples originally screened. The assay that was chosen was the Tumour Response to Antineoplastic Compounds (TRAC) assay (Bosanquet, McCann et al. 1995) due to its conservative requirement for cellular material, and its generation of LC_{90}s using a semi-automated procedure. Thus, in order to support the data generated by PI/FACS, the TRAC assay was used to validate the sensitivity spectrum that had been previously defined. As might be expected, there was a correlation between the two methods, although it was imperfect. This imperfection between the two methods may have been because the TRAC allows visual discrimination of cellular fragments as well as live and dead cells. Furthermore, the timepoints of choice were 2 days apart, and LC_{90}s are of higher stringency than measurement of single-dose killing. Nevertheless, a reasonable correlation was observed between these two assays, thereby supporting the use of the PI/FACS method to define the GC sensitivity of the CLL samples.

Furthermore, GC sensitivity did not correlate with sensitivity to fludarabine, thus suggesting the identification of a resistance mechanism specific to GCs.

To gain further insight into the mechanism and kinetics of Dex-induced killing, further assays were performed. GCs have previously been shown to kill CLL cells through a mechanism that involves apoptosis (Tiwari, Dong et al. 2005, Chandra, Gilbreath et al. 1997, McConkey, Chandra 1999). Markers of apoptosis include PARP cleavage, PS exposure, membrane blebbing, DNA laddering, and mitochondrial perturbation with cytochrome c and SMAC release, and caspase activation (Wang, Malone et al. 2003a, Cohen 1997, Pettitt, Cawley 2000). The former two apoptotic markers were selected for this study as this meant that small amounts of cellular material were required, and the methods had previously been established in this laboratory. It is clear that Dex treatment in the CLL samples tested killed by a mechanism that involved apoptosis, as demonstrated by PS exposure and PARP cleavage. Caspase inhibitors have previously been reported to alter the mode but not the extent of killing of haematopoietic cells (Kroemer, Martin 2005). Nevertheless, to ensure that this was true for GC-induced killing of CLL cells, the effect of a pan-caspase inhibitor on GC-induced killing was tested. As expected, inhibition of caspase activity did not appear to significantly alter the extent of Dex-induced killing, despite the apparent hindrance of mitochondrial depolarisation. Therefore, CLL cells co-treated with GCs and caspase inhibitor possibly die by a mechanism akin to apoptosis that lacks certain hallmarks, or a necrotic mechanism (Section 1.7.1).

As expected from previous findings (Aleskog, Tobin et al. 2004), samples with UM-*IGHV* appeared to respond better to Dex treatment than those with >2% deviation from germline. This could indicate that the nature of BCR signalling or the level of differentiation of a CLL population affects GC sensitivity. Additionally, in opposition to a previous clinical study of CLL (Thornton, Matutes et al. 2003) TP53 deletion was more prevalent in the GC-resistant group, which might suggest that aberrations in factors that can impact upon p53-mediated killing play a role in GC resistance in CLL. It would not appear that previous treatment of patients with chlorambucil sensitised samples to GC within this study. However, the ratio of previously treated to untreated patients was higher in the sensitive group of samples, which possibly suggests a role for previous treatment in GC sensitisation.

Having established an *in-vitro* response to Dex profile for a cohort of CLL samples, the most sensitive and most resistant samples could further be compared and contrasted to establish differences in GC-related signalling between the two sensitivity groups. Many of the effects of GCs at therapeutic levels are mediated by the GC receptor (Schmidt, Rainer et al. 2004), and intact receptor is required for cell killing (Greenstein, Ghias et al. 2002) (Section 1.4.4). Therefore, studies of differences in GC receptor expression and function provided a promising avenue for investigation.

Chapter 3: Glucocorticoid receptor expression and transcriptional activity

3.1 Introduction

In Chapter 2, the relative sensitivity of a panel of CLL samples to Dex-induced killing was determined. Moreover, it was confirmed that GC sensitivity in this cohort does not simply reflect multidrug resistance or a predisposition to spontaneous apoptosis. Furthermore, Dex-induced killing was confirmed to involve apoptosis.

As described in Section 1.5.3, GCs enter the cell by a passive mechanism and then bind to the GR (defined in Section 1.5.2) in the cytoplasm. Molecular rearrangement (Kfir-Erenfeld, Sionov et al. 2010) and hyperphosphorylation leads to exposure of nuclear localisation sequences (De Bosscher, Vanden Berghe et al. 2003). Either a GR-hsp90-FKBP52-dynein complex then migrates to the nucleus, where dissociation can occur to yield GR in the homodimeric DNA-binding form (Davies, Ning et al. 2002, Dittmar, Demady et al. 1997), or GR dimerises in the cytoplasm prior to nuclear translocation (Davies, Ning et al. 2002, Bledsoe, Montana et al. 2002)(Kfir-Erenfeld, Sionov et al. 2010).

GRα is the full-length version of the GR. GRα is responsible for transcription activation (Frankfurt, Rosen 2004) (Section 1.4.5), whilst all other isoforms are truncated versions of GRα and possess altered hormone binding domains, nuclear

localisation sequences, or transactivation domains, and thus are thought to modulate GRα activity (Oakley, Cidlowski 2011). GRβ does not bind GCs, and can act as a dominant negative nuclear inhibitor of activated GRα (Shahidi, Vottero et al. 1999, Lewis-Tuffin, Cidlowski 2006). GRδ is detected in many tissues and has been shown to stimulate the transcriptional activity of GRα in malignant haematological cells (de Lange, Segeren et al. 2001).

As discussed in Section 1.5, GC respsonse may be affected by various aspects of the GR and associated signals. GR isoform expression (Section 1.5.4) and *GR* mutations/polymorphisms (Section 1.5.5), GR post-translational modifications (Section 1.5.6), and aspects of the GR complex have all been reported to play a role in GR transactivation. Therefore, there are many levels at which the GRs of samples from each Dex sensitivity group could be compared and contrasted. With respect to the importance of GR isoform expression in GC-induced killing there is conflicting evidence. In one study, GR levels have been shown to not correlate with *in vitro* sensitivity of CLL cells to GC (Levine, Peterson et al. 1985), whilst in another study, a sample from a GC resistant CLL patient showed markedly reduced GRα expression and high GRβ expression, along with reduced binding affinity for Dex relative to non-malignant lymphocytes (Shahidi, Vottero et al. 1999). With regards to mutations/polymorphisms, a study of CLL cells from GC-treated patients revealed an absence of DNA- and steroid-binding site mutations (Soufi, Kaiser et al. 1995).

Given the importance of the study by Iglesias *et al* (Iglesias-Serret, de Frias et al. 2007) (Section 1.5.7) to the selection of GC-induced genes in CLL, this study will

be briefly described. In MLPA (Multiplex Ligation-dependent Probe Amplification) studies, probes that hybridise to the target sequence are amplified rather than sequences from cDNA samples (Schouten, McElgunn et al. 2002). In contrast to a standard multiplex PCR, a single pair of PCR primers is used for MLPA amplification. The resulting amplification products can be analysed by capillary electrophoresis. Comparing the peak pattern obtained to that of reference samples indicates which sequences show aberrant copy numbers. Gene expression profiling of 9 CLL patient samples by RT-MLPA showed that *Bim* and *FLIP* mRNA were induced (expression approximately doubled) by Dex at 24h. *Mcl1L* was consistently downregulated but to a lesser extent. The RT-MLPA screening panel included members of the Bcl-2 family including BH3-only proapoptotic, Bax-like proapoptotic, and Bcl-2-like antiapoptotic family members, together with members of the IAP family and other miscellaneous apoptosis-related genes. Of particular note, *Bad*, *BMF*, and *NOXA* were not altered in response to Dex at this timepoint.

Given the relevance of the GR to both GC-related signalling (Schmidt, Rainer et al. 2004) and killing (Greenstein, Ghias et al. 2002) (Section 1.4.4), and various evidence from previous studies (Section 1.5), it is possible that qualitative or quantitative defects in the GR and its complex might contribute to a GC resistant phenotype in CLL cells. Furthermore, GC resistance might result from alterations in GR activation via post-translational modifications. Therefore, as a first step in comparing sensitive and resistant samples to elucidate resistance mechanisms, differences in the GR were sought between the two groups.

3.2 Materials and Methods

3.2.1 Cell isolation

Peripheral blood mononuclear cells were isolated and stored as described in Section 2.2.3.

Blood samples from patients with high neutrophil counts were acquired and Polymorphprep (Nylomed Pharma AS, Oslo) was used to harvest the neutrophils according to the manufacturer's instructions.

3.2.2 CLL cell culture

Cells were thawed, and cultured at densities of $5x10^6$ cells/ml under the conditions described in Section 2.2.5, for 6h/17.5h/21.5h.

3.2.3 Preparation of protein samples and Western blotting

Samples were harvested and lysed in SDS Sample Buffer, containing 125mM Tris-HCl (pH6.8), 20% glycerol (Sigma), 4% SDS (Fisher Scientific, Loughborough, Leicestershire, UK), 10% β-mercaptoethanol (Sigma) and 0.006% bromophenol blue (VWR International, Lutterworth, Leicestershire, UK).

Western blotting was performed as in Section 2.2.8, using the antibodies specified in Table 3.2.1.

Table 3.2.1 Antibodies used in immunoblotting. Antibody incubations were for 1h at R/T or O/N at 4°C.

Primary Antibody	Dilution	Secondary Antibody	Dilution
GR Rabbit PAb Santa Cruz	1/1000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/2000
GRβ Rabbit PAb Abcam	1/500 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/1000
β-Actin Mouse PAb Sigma	1/10000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000

3.2.4 RNA reverse transcription to cDNA

Total cellular RNA was extracted from up to 5 x10^6 cells using the RNeasy kit and shredder columns according to the manufacturer's instructions (Qiagen, Crawley, Sussex, UK), and was then reverse transcribed using an oligo$(dT)_{15}$ primer with MMLV reverse transcriptase (Promega Southampton, Hampshire, UK). All of the RNA preparations showed a 260nm/280nm OD ratio of at least 1.6. 2μg of RNA in 25μl were mixed with 2μl of the oligo$(dT)_{15}$ primer, and heated at 70°C for 5min. The samples were transferred to ice and then 1μl RNAsin, 8μl 5x RT buffer, 2μl dNTPs, and 2μl of MMLV-RTase were added. Following a 1h reverse transcription at 37°C, and a 10min 65°C deactivation step, the cDNA samples were frozen at -20°C.

3.2.5 Standard PCR

1μl of cDNA was used in a 50μl PCR to amplify the regions of interest using 0.5μl Taq polymerase and the supplied polymerase buffer (Promega), 3mM MgCl, 0.2mM dNTPs and up to 0.4pmol/μl of both forward and reverse primers (listed in

table 3.2.2). PCR products were then separated by electrophoresis on 1-2% agarose gels in 0.5 x TBE buffer (Sigma), stained with ethidium bromide (EtBr) and visualised with UV.

GRβ was measured by standard PCR using 0.4pmol/µl of forward and reverse primer, and an annealing temperature of 61°C. The resulting product was 393bp in length.

Bim-EL/L/S were detected by standard PCR using 0.4pmol/µl of forward and reverse primer, and an annealing temperature of 61°C. The resulting products were 481bp (*EL*), 312bp (*L*), and 233bp (*S*) nucleotides in length.

β-Actin (*ACTB*) was detected by standard PCR using 0.4pmol/µl of forward and reverse primer, and an annealing temperature of 61°C. The resulting product was 626bp in length.

3.2.6 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

All real-time PCRs were carried out in triplicate using an Opticon 2 thermal cycler (MJ Research, Waltham, Mass., USA), or in duplicate using a Stratagene Mx3005P PCR machine (Agilent, Stockport, UK), alongside no-template controls for each set of primers. Programs consisted of a 10min 95°C melt step, followed by up to 44 cycles of 30s at 94°C, 30s at the appropriate annealing temperature, and a 72°C extension step of 30s. A melt curve was performed from 55°C to 95°C, following a 30s melt step. A 10min extension step completed the program.

Each 25µl reaction included 12.5µl of either DyNamo Master Mix (Finnzymes, Espoo, Finland) or Applied Biosystems Power SyBr Green (Applied Biosystems, Warrington, UK) (including Tbr polymerase, SYBR Green I, 5mM Mg^{2+} and dNTPs), and up to 0.4pmole of each relevant forward and reverse oligonucleotide primer. 0.5µl stock cDNA was used in each 25µl (1/50) reaction. Following the extension step of each cycle, the fluorescence level was measured at 76-83°C.

The relative levels of transcripts were normalised to those of *ACTB* or *ribosomal protein L27* (*RPL27*). Arbitrary expression levels were calculated from the number of cycles taken to reach a fixed fluorescence intensity (threshold) during the linear phase of amplification (C_t values). The formula "power(2,-(C_t^{target}-$C_t^{normaliser}$)" was applied to yield a relative quantity of transcript of each target gene in each sample.

Table 3.2.2 displays real-time PCR conditions for amplification of each gene studied.

Table 3.2.2. qRT-PCR conditions for target genes.

Target	Primer Concentration (pmol/µl)	Master Mix	Anneal Temp (°C)	Read Temp (°C)	Product Length (bp)
ACTB	0.4	Dynamo	59	85	626
GRα	0.16	Dynamo	60	78	408
GRδ	0.4	Applied Biosystems	57	72	365
RPL27	0.4	Dynamo	60	79	345
Bim-EL	0.4	Applied Biosystems	58	83	355
GILZ	0.24	Dynamo	60	81	401
GAPDH	0.4	Dynamo	60	82	452

Primer sequences for PCR are listed below.

ACTB

Actin For: 5'-CCTCGCCTTTGCCGATCC - 3'

Actin Rev: 5'- GGATCTTCATGAGGTAGTCAGTC-3'

GRα

GR For 2: 5'-CTGTGTTTTGCTCCTGATCTG-3'

GR Rev α 2: 5'-GATTTCAGCTAACATCTCGGG-3'

GRδ/GRβ

GR For : 5'-TGTTTTGCTCCTGATCTGA-3'

GR Rev δ: 5'-GTTTCTGCCATACCTATTTG-3'

GR Rev β: 5'-TGAGCGCCAAGATTGTTG-3'

RPL27

RPL27 For: 5'-GACGCAAAGCTGTCATCGTG-3'

RL27 Rev: 5'-GCAGTTTCTGGAAGAACCAC-3'

Bim-EL/BIM-EL/L/S

BIM-EL-F: 5'-AATCCTGAAGGCAATCACGGA-3'

BIM-F1: 5'-ATGGCAAAGGAACCTTCTGATG-3'

BIM-R5: 5'-CGTTAAACTCGTCTCCGATAC-3'

GILZ

GILZ 1 F: 5'-ACCGAAATGTATCAGACCCCC-3'

GILZ 1 R: 5'-ACTTACACCGCAGAACCACCA-3'

GAPDH

GAPDH For: 5'-ACCACAGTCCATGCCATCAC-3'

GAPDH Rev: 5'-TCCACCACCCTGTTGCTGTA-3'

Each PCR product could be detected as a single band on agarose gels following the number of cycles sufficient to reach the designated thresholds for quantitation by qRT-PCR (Figure 3.2.1.1 A/3.2.1.2 A). Amplification plots display the threshold (horizontal lines), placed to intercept the linear phase of product amplification (Figure 3.2.1.1 B/3.2.1.2 B). Dissociation curves for qRT-PCR products display segregation between primer dimer and product peaks, allowing quantitation by placement of read temperature beyond the melting point of the primer dimer (Figure 3.2.1.1 C /3.2.1.2 C).

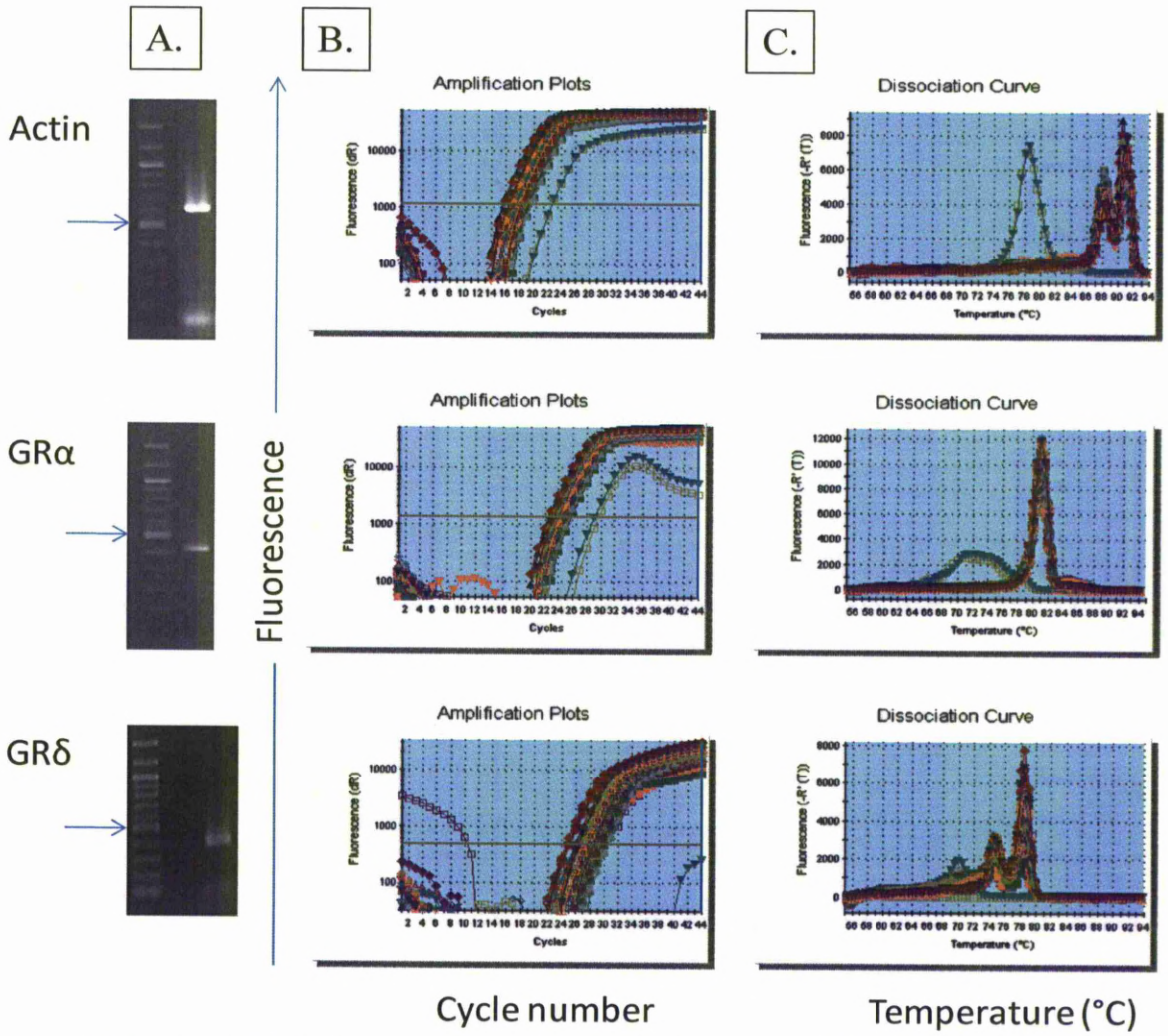


Figure 3.2.1.1 qRT-PCR products for *ACTB*, *GRα*, and *GRδ* are of the expected size, and can be reliably measured by qRT-PCR. A. Following 44 cycles of QRT-PCR, products (right lane) were separated on 1% agarose gels alongside 100bp marker (left lane). Blue arrows highlight 500bp marker. Product sizes were: 626bp for *ACTB*, 408bp for *GRα* and 365bp for *GRδ*. B. Amplification plots show threshold placement based upon amplification profile. C. Dissociation curves show segregation of primer dimer from product peak, which allowed measurement of specific product abundance.

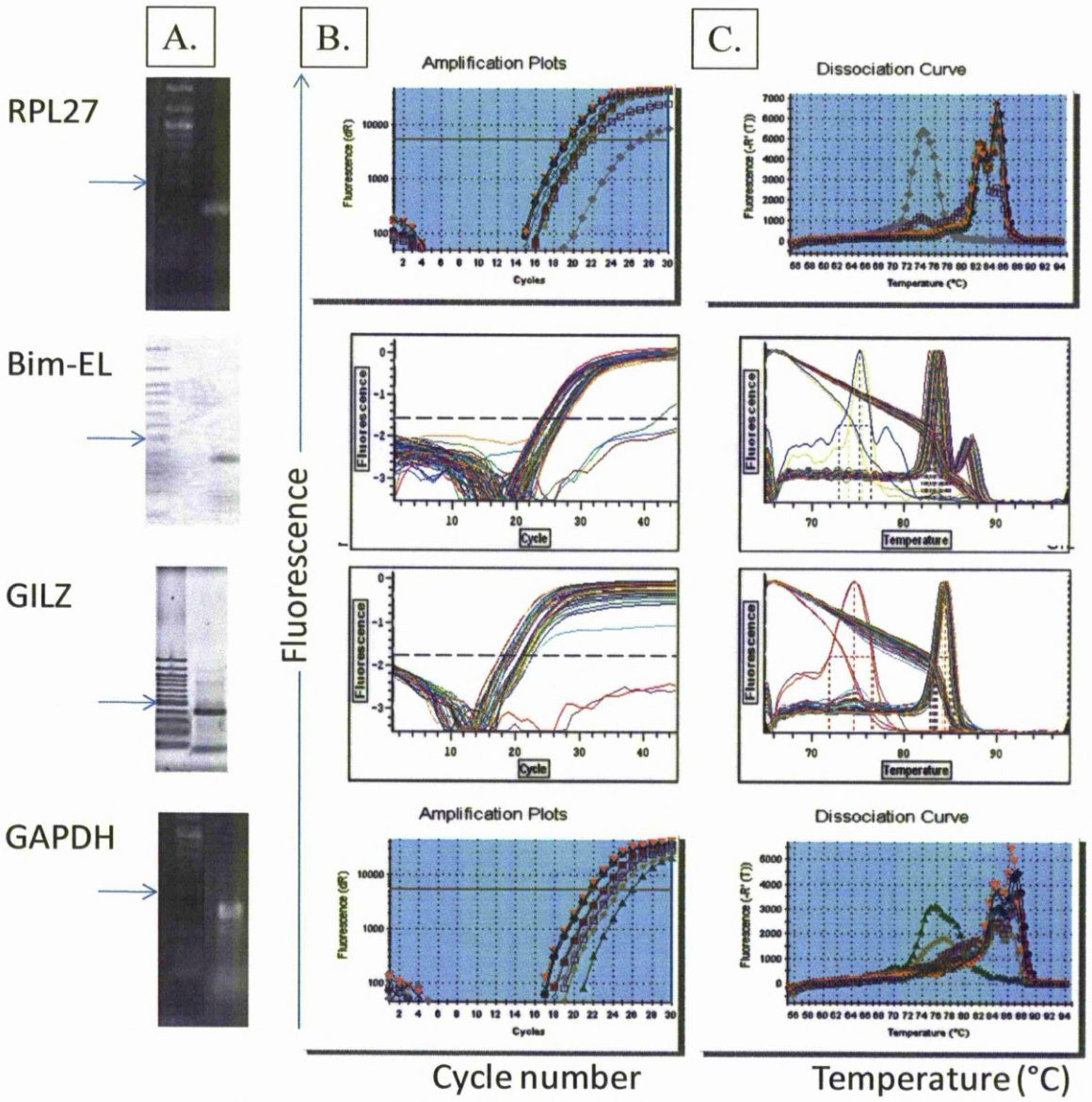


Figure 3.2.1.2 qRT-PCR products for *RPL27*, *Bim-EL*, *GILZ*, and *GAPDH* are of the expected size, and can be reliably measured by qRT-PCR. A. Following 44 cycles of qRT-PCR, products (right lane) were separated on 1% agarose gels alongside 100bp marker (right lane). Blue arrows highlight 500bp marker. Product sizes were: 345bp for *RPL27*, 255bp for *Bim-EL*, 401bp for *GILZ* and 452bp for *GAPDH*. B. Amplification plots show threshold placement based upon amplification profile. C. Dissociation curves show segregation of primer dimer from product peak, which allowed measurement of specific product abundance.

3.3 Results

3.3.1 Basal expression of key GR isoforms

It was first important to identify whether major differences in GR levels existed between sensitive and resistant samples. Expression of GRα, GRδ, and GRβ in particular is relevant to CLL (Oakley, Cidlowski 2011, Shahidi, Vottero et al. 1999, de Lange, Segeren et al. 2001) (Section 1.5.4/1.5.5), and so the baseline mRNA expression of each isoform was measured to identify whether expression of GR isoforms in the samples of this study was associated with sensitivity to Dex.

GRα mRNA levels were highest in certain sensitive samples, but the median expression for each sensitivity group was very similar ($P>0.05$) (Figure 3.3.1). Contrastingly, sensitive samples appeared to express higher *GRδ* mRNA than resistant samples ($P<0.05$). However, this corresponded to only an approximate doubling of the median expression between resistant and sensitive samples. Therefore, in keeping with a previous study in CLL (Levine, Peterson et al. 1985), *GRα* mRNA expression at baseline was probably not associated with GC sensitivity, whilst *GRδ* mRNA expression at baseline may have been involved.

GRβ differs from GRα and GRδ in that it can act as a suppressor of GR heterodimer function (Shahidi, Vottero et al. 1999, Lewis-Tuffin, Cidlowski 2006). Therefore, it would be expected that if the baseline expression of this isoform were significantly different between sensitive and resistant samples then the resistant samples would contain the highest levels. Neutrophils contain relatively high levels of GRβ (Strickland, Kisich et al. 2001), and so cDNA from

neutrophils served as a positive control for optimisation. *GRβ* mRNA baseline expression was found to be almost undetectable in sensitive and resistant CLL samples (Figure 3.3.2). In contrast, a positive control neutrophil sample showed a significant level of expression by standard PCR. Therefore, *GRβ* mRNA expression at baseline did not support an important role for GRβ in GC resistance in this study. This is perhaps unsurprising given that the concentration of *GRα* mRNA in CEM-C7, a T-cell acute lymphoblastic leukaemia cell-line, is 100-fold higher than that of *GRβ* mRNA (Pedersen, Vedeckis 2003).

To better define the expression of the various GR isoforms it was critical to look at baseline protein expression. At the time of this investigation, there was no commercially available GRδ antibody. Though *GRβ* mRNA expression was not quantifiable in CLL samples (Figure 3.3.2), it was possible to measure protein levels. Therefore, levels of GRα and GRβ protein alone were measured using Western blotting (Figure 3.3.3 A).

In keeping with the mRNA data (Figure 3.3.1), there was no clear difference in protein expression of the GRα isoform between sensitive and resistant samples ($P>0.05$) (Figure 3.3.3 B). In the case of the antagonistic GRβ isoform there was also no significant difference between the two groups of samples ($P>0.05$). This finding is in contrast with a previous study of GC resistance in CLL (Shahidi, Vottero et al. 1999), but is supported by a separate study (Levine, Peterson et al. 1985). It was concluded that the baseline protein expression of GRα and GRβ were unlikely to be responsible for the failure of Dex-induced apoptosis in resistant samples.

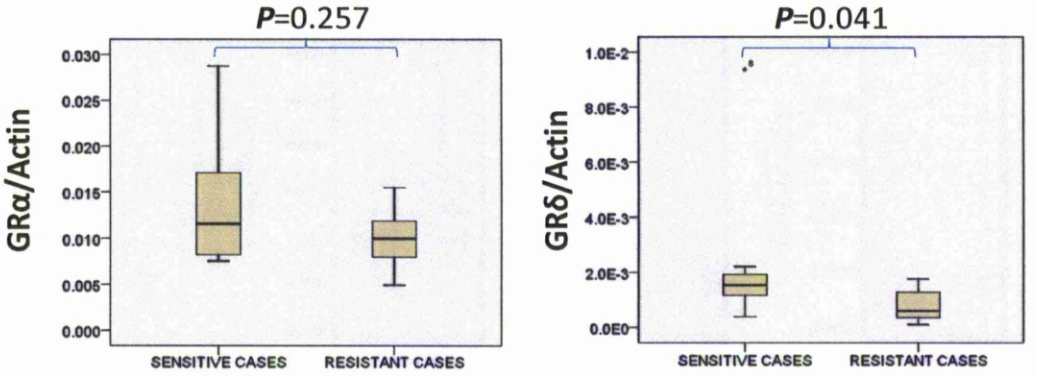


Figure 3.3.1. Baseline expression of *GRα* mRNA does not correlate with Dex sensitivity group, whilst expression of *GRδ* mRNA appears to be higher in sensitive than resistant samples (10 sensitive vs. 10 resistant). qRT-PCR was performed to quantify expression of *GRα* and *GRδ* mRNA. All target quantities were normalised to Actin, which acted as a loading control. ***P*-values** represent Mann-Whitney *U* tests for group difference: *GRα* ***P***=0.257; *GRδ* ***P***=0.041.

Figure 3.3.2. *GRβ* mRNA is detectable by standard PCR in neutropil but not CLL samples. Equal concentrations of neutrophil cDNA and that of a sensitive and resistant CLL sample were used in standard PCR to amplify *GRβ* mRNA using an annealing temperature of 61°C. The resulting products were separated on 1% agarose gels alongside 100bp marker (left lane). The *GRβ* product resolved at 398bp, as expected.

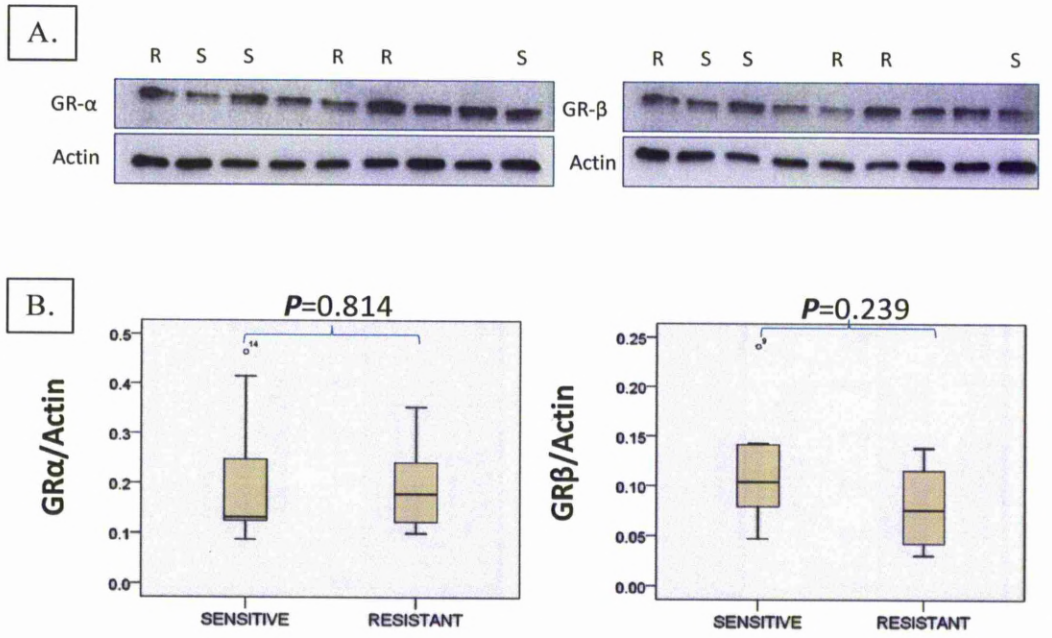


Figure 3.3.3. Neither baseline protein expression of GRα nor GRβ correlates with GC sensitivity (9 sensitive vs. 6 resistant samples). A. Representative Western blots for GRα (left) and GRβ (right). Sensitive samples (S) and resistant samples (R) are highlighted. B. Relative quantity of each protein to Actin was evaluated by densitometry. ***P***-values represent Mann-Whitney *U* tests for group difference: GRα ***P***=0.814; GRβ ***P***=0.239.

3.3.2 Transcriptional readout validation

There is much evidence to suggest that the GR contributes to GC-induced killing by regulating gene transcription, as described in Section 1.5.7. Hence, a diverse set of gene expression changes have been observed in leukaemic cell types. These include genes that block proliferation, cause growth arrest, and regulate apoptosis (Schmidt, Rainer et al. 2006, Yoshida, Miyashita et al. 2002); and genes that impact upon MAPK pathways, NF-κB signalling, and carbohydrate metabolism (Tissing, den Boer et al. 2007). Small sets of genes have been identified that correlate with GC sensitivity in cells from several hematologic malignancies (Miller, Komak et al. 2007). These included both *Bim*, a proapoptotic member of the BH3-only group of Bcl-2 family proteins; and *GILZ*, a target with numerous roles that include NF-κB antagonism. *Bim* mRNA and protein induction by GCs has been demonstrated in several ALL models (Wang, Malone et al. 2003a, Lu, Quearry et al. 2006, Ploner, Rainer et al. 2008, Abrams, Robertson et al. 2004, Erlacher, Michalak et al. 2005, Bachmann, Gorman et al. 2007) and primary CLL cells (Iglesias-Serret, de Frias et al. 2007). *GILZ* mRNA has been shown to be induced by GCs in T cells and ALL models (Mittelstadt, Ashwell 2001, Bachmann, Gorman et al. 2007).

It has been suggested that the scale and duration of GR expression can influence GC-induced signalling (Section 1.5.5). In T cells and other cell types sensitive to GC-induced death, positive autoregulation is commonly observed, and sometimes a requirement (Eisen, Elsasser et al. 1988)(Ramdas, Liu et al. 1999)(Ashraf, Kunapuli et al. 1991)(Barrett, Vig et al. 1996)(Gomi, Moriwaki et al. 1990)(Pedersen, Geng et al. 2004). Despite this evidence for autoinduction, *GRα*

transcript expression has been shown to fall in response to Dex in CLL cells (Meyers, Taverna et al. 2007).

Though baseline expression of GR isoforms was similar between the two groups of samples, a functional study of the capacity of GR to transcribe target genes would provide direct functional evidence to test whether the GRs of the two groups of samples differed significantly in their ability to initiate transcription. If the GR of sensitive samples but not resistant samples could be shown to regulate target genes in response to Dex, it would be clear that studies of the GR and its regulation would be required to pin-point GR-related defects in resistant samples. If no clear difference in transcription of target genes existed between the two groups, the investigation would move downstream beyond GR transcriptional activity. Therefore, mRNA expression of *Bim*, *GILZ*, and *GRα* were used to measure the integrity of GR-mediated transcription of pro-death genes.

Bim exists as three major splice variants in lymphoid cells (Abrams, Robertson et al. 2004). In order to quantify *Bim* transcript by qRT-PCR, it was first necessary to demonstrate that measurement of a single *Bim* mRNA transcript could be used to represent *Bim* mRNA *per se*. This could be achieved by showing that the ratio of expression of the three dominant isoforms was similar with and without Dex treatment, using primers that could amplify all three isoforms by standard PCR. By amplifying *Bim* for only 25 cycles (close to the exponential phase of amplification) it was possible to estimate transcript abundance. It appeared that the three *Bim* isoforms were both detectable and Dex-inducible (Figure 3.3.4). Moreover, the ratio of *Bim* mRNA expression between the three dominant

isoforms was similar with and without Dex treatment (Figure 3.3.4). Therefore, measurement of *Bim-EL* expression could be used to represent *Bim*.

Prior to screening samples to identify whether target genes were upregulated, it was important to select an appropriate gene for normalisation of qRT-PCR data, as many commonly used normalising genes vary between experimental conditions as well as cell types (de Jonge, Fehrmann et al. 2007). It was expected that some change in normaliser expression would occur post-treatment, as sample quality might be compromised by the initiation of killing. However, as GCs can impact upon up to 20% of the human leukocyte genome (Galon, Franchimont et al. 2002), care had to be taken to not choose a normaliser that was itself being considerably altered by GR action. Within this research group, three commonly used housekeeping genes for quantitative PCR include *ACTB* (structural), *GAPDH* (glycolysis), and *RPL27* (ribosomal). The use of *ACTB* as a normaliser was rejected due to the substantial downregulation of this gene upon Dex treatment (Figure 3.3.5A). Though changes were also detected in *GAPDH*, and *RPL27* (Figure 3.3.5A), comparison of expression of these two housekeeping genes correlated fairly well (Figure 3.3.5B). As *RPL27* displayed a smaller range of Dex-induced expression change than *GAPDH* (Figure 3.3.5B), and produced a melt curve of higher integrity by qRT-PCR (Figure 3.2.1.2), this gene was selected as the housekeeping gene for further studies.

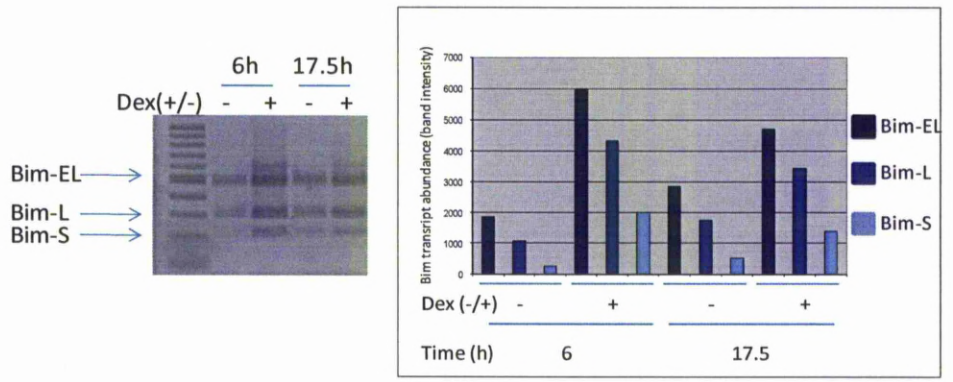


Figure 3.3.4. *Bim* mRNA expression appears to be induced in CLL cells in response to Dex, and the expression of *Bim-EL*, *Bim-L*, and *Bim-S* appear to strongly correlate. CLL samples were cultured w/wo 100μM Dex for 6h and 17.5h. *Bim* was amplified from resulting cDNA samples by 25 cycles of standard PCR. PCR products were separated by agarose gel electrophoresis and visualised with ethidium bromide, which allowed quantity determination via densitometry.

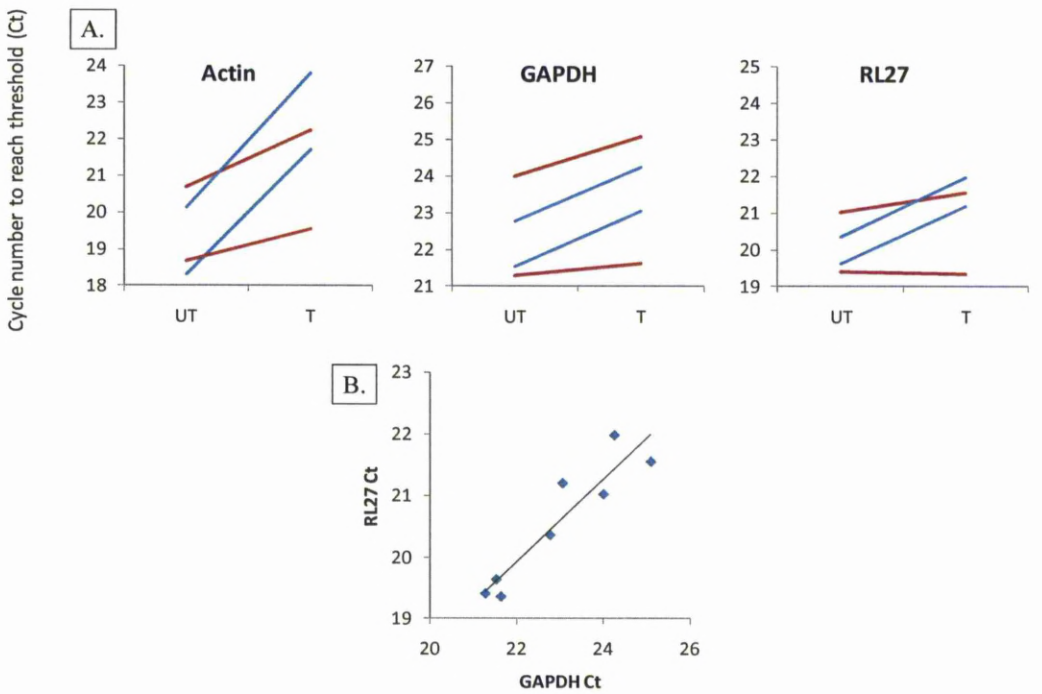


Figure 3.3.5. Of a panel of potential normalising genes, *RPL27* appears to be altered least between samples incuabetd w/wo Dex. Blue lines = sensitive samples; red lines = resistant samples. A. Number of cycles required to reach threshold (C_t) for 2 sensitive (blue) and 2 resistant (red) samples cultured for 21.5h w/wo 100nM Dex. B. *GAPDH* vs. *RL27* C_t.

3.3.3 Transcriptional readout of GR activity

Timepoints selected for measurement of mRNA expression were 0h, 6h, and 21.5h in order to provide both an indication of early alterations in gene expression mediated by GCs, and to provide data relating to an intermediate timepoint between 6h and 48h (the timepoint used to define sample sensitivity groups). These timepoints were in keeping with those used previously in studies of the effects of GCs on CLL cells and an ALL cell line (Lu, Quearry et al. 2006, Iglesias-Serret, de Frias et al. 2007). More specifically, an approximate doubling of *Bim* mRNA abundance by 24h and close to maximum Bim protein expression at both 24h and 48h has been observed upon GC treatment of CLL cells (Iglesias-Serret, de Frias et al. 2007). *Bim* mRNA has been shown to be significantly induced by between 8h and 16h after GC treatment in an ALL cell line, which was maintained until up to 24h, while Bim protein induction was shown as early as 16h (Lu, Quearry et al. 2006). Significant *Bim* induction has also been observed in ALL cells in response to Dex over a 24h incubation, which preceded the onset of death (Wang, Malone et al. 2003a). Generally, studies of mRNA changes in response to GCs in leukocytes measure expression within a 24h period (Section 1.5.7).

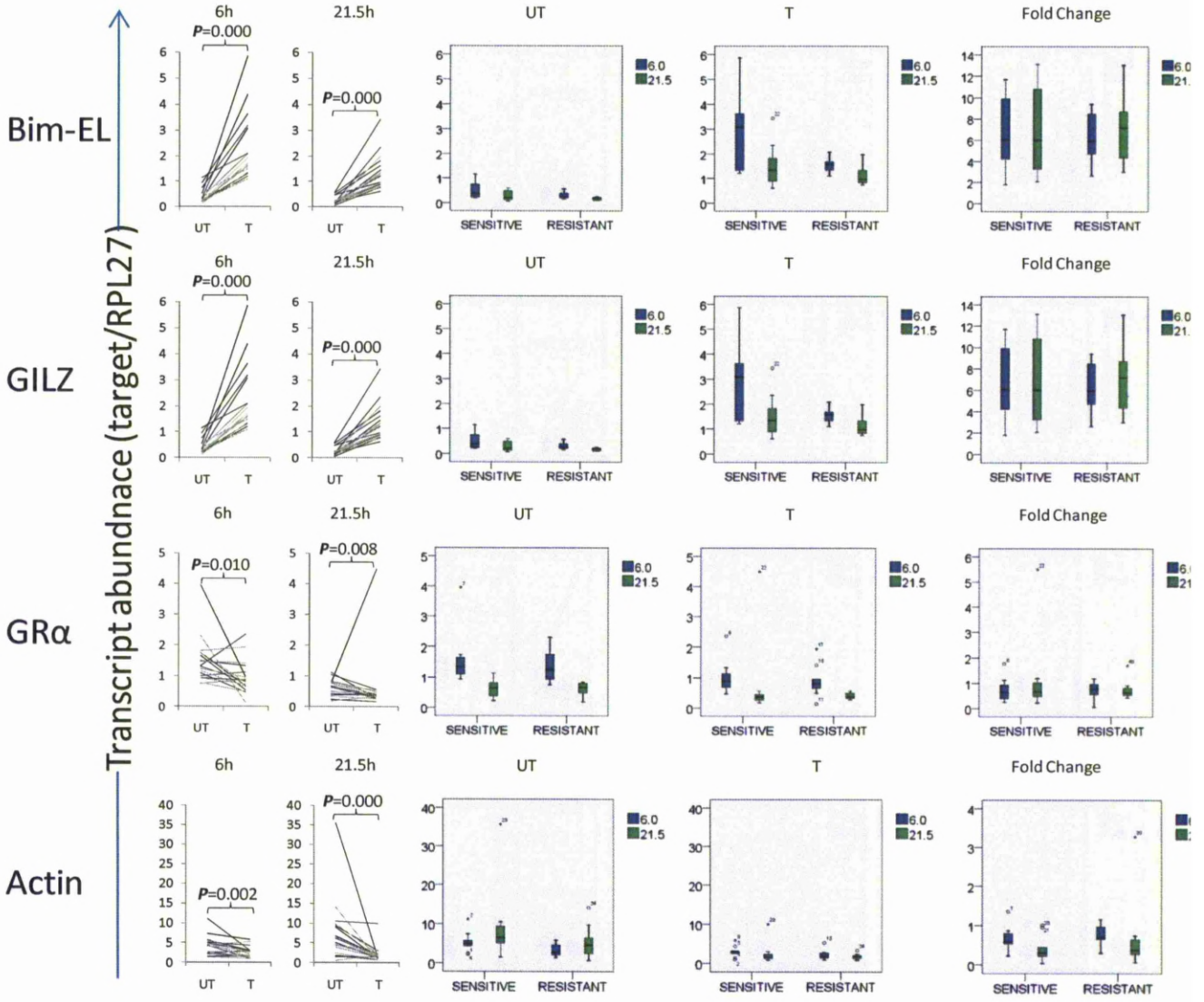


Figure 3.3.6. Resistance of CLL cells to GC treatment does not appear to result from a failure to transcribe proapoptotic target genes or downregulate *ACTB*. Samples were cultured w/wo 100nM Dex for 6 (n=9S/9R) and 21.5h (n=10S/10R). cDNA was used to quantify transcript abundance by qRT-PCR of *Bim-EL*, *GILZ*, *GRα,* and *ACTB*, relative to *RPL27*. In line charts, black lines = sensitive samples, whilst grey lines = resistant samples. *Bim* and *GILZ* appear to be significantly induced at 6h and 21.5h (Wilcoxon ***P***<0.000/0.000), whilst *GRα* (Wilcoxon ***P***=0.01/0.008) and *ACTB* (Wilcoxon ***P***=0.002/0.000) expression is significantly downregulated. Stem and leaf plots show untreated (UT), Dex treated (T), and fold change expression of each transcript relative to *RPL27* by sensitivity group and timepoint (blue bars = 6h, green bars = 21.5h).

Table 3.3.1. *P*-values for statistical comparisons of expression and fold change of GR-regulated genes between sensitive and resistant samples show no significant differences. *P*-values represent Mann-Whitney *U* tests for group versus group difference for data displayed in Figure 3.3.6.

	6h			**21.5h**		
	Untreated	**Treated**	**Fold change**	**Untreated**	**Treated**	**Fold change**
Bim-EL	0.691	0.102	0.122	0.131	0.226	0.88
GILZ	0.122	0.085	0.627	0.257	0.29	0.496
GRα	0.691	0.627	0.757	0.940	0.545	0.762
ACTB	0.093	0.122	0.31	0.226	1	0.496

Table 3.3.2 Group median expression and fold change of GR-regulated genes

	Untreated				**Treated**				**Fold change**			
Timepoint	6h		21.5h		6h		21.5h		6h		21.5h	
Sensitivity	S	R	S	R	S	R	S	R	S	R	S	R
Bim-EL	1.2	1.4	0.8	0.6	6.6	3.2	2.1	1.5	6.6	2.8	3.0	2.4
GILZ	0.4	0.2	0.2	0.2	3.1	1.6	1.3	0.1	6.0	5.9	6.0	7.2
GRα	1.3	1.2	0.6	0.7	0.9	0.8	0.4	0.4	0.6	0.8	0.7	0.6
ACTB	4.8	2.7	6.4	4.5	3.0	1.7	1.6	1.7	0.6	0.7	0.3	0.4

In accordance with a previous study of GC-treated CLL cells (Iglesias-Serret, de Frias et al. 2007) and Figure 3.3.4, *Bim-EL* expression was upregulated by Dex in the CLL samples at both 6h and 21.5h (P<0.000) (Figure 3.3.6). Also in keeping with previous studies of T cells and ALL models (Mittelstadt, Ashwell 2001, Bachmann, Gorman et al. 2007), *GILZ* was induced by Dex at both timepoints (P<0.000). *GRα* transcript was downregulated in most samples at 6h and 21.5h (P=0.01/0.008), which was consistent with a previous study of CLL cells (Meyers, Taverna et al. 2007). *ACTB* also appeared to be downregulated in most samples at both timepoints (P=0.002/0.000).

The line charts and stem-leaf plots of Figure 3.3.6 suggest a tendency for Dex to upregulate *Bim-EL* and *GILZ* at 6h more in sensitive than resistant samples, which implies a difference between the two groups in terms of GR-transactivation capacity. In fact, five sensitive samples displayed induction at 6h of a greater magnitude than that observed in any resistant case for both *Bim-EL* (median expression after Dex treatment 6.6 versus 3.2) and *GILZ* (median expression after Dex treatment 3.1 versus 1.6) (Table 3.3.2). Furthermore, median Dex-induced fold change in *Bim-EL* expression at 6h was 6.6 for the sensitive group, and less than half this value, 2.8, for the resistant group. However, when the statistics were considered (Table 3.3.1) all hypotheses regarding differences in baseline, Dex-induced or fold change expression were rejected (P>0.05). Furthermore, the two groups displayed very similar profiles of expression of all targets at 21.5h (Figure 3.3.6), and median fold change in *GILZ*, *GRα* and *ACTB* expression was very similar at both timepoints (Table 3.3.2). Taken together, these observations

suggest that failure of GC-induced alteration of target genes is not predominantly responsible for resistance to Dex (D'Adamio, Zollo et al. 1997).

3.4 Discussion

The aim of this chapter was to compare GC sensitive and GC resistant CLL samples for GR expression and function.

To address the first of these aims, baseline expression of the three major lymphoid GR isoforms (Oakley, Cidlowski 2011)(Shahidi, Vottero et al. 1999, Lewis-Tuffin, Cidlowski 2006)(de Lange, Segeren et al. 2001) was measured at the mRNA and protein level. All comparisons between sensitive and resistant samples suggest that no differences existed between the two groups in terms of baseline *GR* mRNA and protein expression, apart from mRNA expression of *GRδ*, which was more highly expressed at baseline in sensitive samples, though by only 1-fold. This finding indicates that insufficient expression of GRδ might contribute to the resistance of CLL cells to Dex treatment, as GRδ has been reported to positively contribute to GR-mediated signalling (de Lange, Segeren et al. 2001). However, this could not be confirmed at the protein level due to the lack of a suitable antibody, and so this aspect of the study may be worth developing in the future. As GRα mRNA and protein levels were similar between the two sensitivity groups, differences in the expression of this isoform could not account for Dex resistance in this study. GRβ protein expression was not associated with sensitivity, and *GRβ* mRNA levels were sub-quantifiable in CLL samples. Various studies could have been performed to identify receptor expression over time, and to test whether Dex treatment had an impact upon protein expression level. However, due to the multiple possible differences in GR expression and regulation that might exist (Section 1.5), it was decided to study

the capacity of the GR to regulate target genes in the two sensitivity groups in the first instance.

To assess functionality of the GR, induction of proapoptotic genes that are reported to be GC induced in lymphoid cells (Ramdas, Liu et al. 1999, Mittelstadt, Ashwell 2001, Iglesias-Serret, de Frias et al. 2007, Bachmann, Gorman et al. 2007) were used as a readout of GR signalling capacity. Additionally, samples were screened for *ACTB* expression as this was originally a potential normalising gene for qRT-PCR (Figure 3.3.5). *Bim-EL* and *GILZ* were upregulated by Dex treatment in both sensitive and resistant samples. *GRα* was generally downregulated in samples of both sensitivity groups, suggesting that autoregulation of the GR is not critical in GC-induced killing of CLL cells. *ACTB* was downregulated by Dex treatment in samples of both groups, which perhaps indicates a role for GCs in dysregulation of the cytoskeleton in CLL.

Careful examination of the qPCR functional readout data showed that a handful of sensitive samples upregulated *Bim-EL* and *GILZ* beyond the level reached by any resistant case. This suggested that there was a tendency for sensitive samples to show higher GR pro-death transcriptional activity than resistant cases. It may have been interesting to focus on the group of sensitive samples that showed the highest induction of *Bim-EL* and *GILZ* for further studies of the receptor and its activation, and future work in this area would be justified. Also, as transcriptional regulation is often dynamic, the measurement of a more thorough timecourse in expression of the target mRNAs may have been useful. Nonetheless, statistically the qPCR functional readout data rejects the hypothesis that a GR-mediated

transactivation defect exists in resistant samples. Moreover, the GR of resistant samples appeared to transcribe targets in response to Dex that were shown to be upregulated in sensitive samples.

The aim of this investigation was to identify key aberrations in GC-induced killing in resistant samples. As GR-related defects did not appear to be responsible for GC resistance of CLL samples, it was decided to examine events downstream, beyond GR transcription. To begin, it was decided that a parallel level of GR signalling in the form of NF-κB signalling would be studied. NF-κB is both an important mediator of CLL survival (Furman, Asgary et al. 2000) and a known GR antagonist (Ray, Zhang et al. 1995) (Section 1.6).

Chapter 4: Glucocorticoid receptor cross-talk

4.1 Introduction

The previous chapter showed that defects in GR-mediated transcription are unlikely to predominantly responsible for GC resistance in CLL cells. Therefore, parallel and downstream signalling pathways were considered for further exploration.

The GR interacts with a number of cytokine-induced transcription factors and members of the nuclear hormone receptor superfamily, including NF-κB, AP-1, NF-AT, CREB, STAT, and Oct (Refojo, Liberman et al. 2001, Gottlicher, Heck et al. 1998) (Section 1.6.1). Of the key survival signalling pathways in CLL, NF-κB (Section 1.6.2) is both constitutively active *in vitro*, and has been shown to be essential to maintaining viability (Cuni, Perez-Aciego et al. 2004, Hewamana, Alghazal et al. 2008). Moreover, NF-κB provides a direct link with the GR as the GR can compete for NF-κB promoters, or co-activator proteins such as CBP/p300, and induce post-translational modifications (De Bosscher, Vanden Berghe et al. 2003, Greenstein, Ghias et al. 2002), and can also directly associate with the p65 subunit (Scheinman, Gualberto et al. 1995, Nissen, Yamamoto 2000, Ray, Zhang et al. 1995, Adcock, Nasuhara et al. 1999) (Section 1.6.4). GR can also transactivate GILZ (D'Adamio, Zollo et al. 1997), as has already been indicated (Figure 3.3.6), and IκBα (Auphan, DiDonato et al. 1995, Scheinman, Cogswell et al. 1995). GILZ and IκBα have both been shown to repress NF-κB activity (Yang,

Zhang et al. 2008, Ayroldi, Zollo et al. 2007)(De Bosscher, Vanden Berghe et al. 2003) (Section 1.6.3/1.6.5). Critically, NF-κB has been shown to be downregulated in response to Dex in CLL (Furman, Asgary et al. 2000) cells, which supports a role for NF-κB in GC-induced killing.

As described in Section 1.6.2, the principle NF-κB proteins in CLL are p65, p50, and c-Rel DNA, as their promoter binding activity has been consistently demonstrated in CLL, and p65 has been strongly linked with *in vitro* survival of CLL cells, as well as disease progression (Furman, Asgary et al. 2000, Hewamana, Alghazal et al. 2008, Schattner 2002). Furthermore, a physical interaction between the GR and p65 and p50 has previously been demonstrated *in vitro*, though only p65 can reciprocally repress GR activity as p50 lacks a transactivation domain (Scheinman, Gualberto et al. 1995, Wissink, van Heerde et al. 1997). p65:p50 heterodimers are the most commonly observed form of NF-κB in B cells (Sen 2006). Interestingly, p50 homodimers, which lack classical transcription activation domains, can obstruct Rel complexes that feature active p65 (Sen 2006). NF-κB pro-survival, growth and proliferation target genes include: *Bcl-xl, Bcl-2A1, Bcl-2, c-FLIP, Bcl-w XIAP, c-IAP, c-Myc, c-Myb, Cyclins D1 and D2,* and the cytokines *IL-2, IL-6, and CD40L* (Horie, Watanabe et al. 2006, Vallabhapurapu, Karin 2009). Given the importance of some of these targets in CLL cell survival, it is not surprising that NF-κB activity has been positively correlated with *in vitro* survival of CLL cells (Hewamana, Alghazal et al. 2008).

Regulation of NF-κB is described in Section 1.6.3. The central mechanism underlying NF-κB regulation is the proteolytic degradation of the family of IκBs (Neumann, Naumann 2007). This "canonical" pathway of NF-κB activation involves phosphorylation of IκBα by IKKβ, which prevents IκBα from repressing NF- κB. NF-κB activates the promoter of IκBα and thus its expression is subject to an autoregulatory negative feedback loop. NF-κB activity can also be regulated via inhibitory post-translational modification, especially phosphorylation, of Rel subunits via the non-canonical pathway (Neumann, Naumann 2007).

Lck is a BCR-associated kinase that regulates BCR signalling (Section 1.6.6). *Lck* mRNA expression has been positively correlated with Dex resistance in CLL cells, and its inhibition has been shown to enhance GC sensitivity (Harr, Caimi et al. 2010). Therefore, elevated Lck protein expression, which is yet to be reported, might play a role in the resistance of the cohort used in this study. GSK-3α/β is a serine/threonine kinase that acts a variety of susbstrates (Section 1.6.6). GSK-3 activity has been recently reported as a factor that can contribute to GC sensitivity in haematopoietic cancer cells (Kfir-Erenfeld, Sionov et al. 2010). Phosphorylation of Ser_{404} of the GR by GSK-3β can protect against Dex killing in U-2 OS cells (Galliher-Beckley, Williams et al. 2008), and GSK-3 inhibition in T cells has been shown to inhibit Bim-induced killing (Spokoini, Kfir-Erenfeld et al. 2010). In CLL cells, GSK-3β prevents epigenetic silencing of NF-κB-mediated gene transcription and thus maintains cell viability and prosurvival Bcl-2 and XIAP expression (Ougolkov, Bone et al. 2007). Therefore, differences in GSK-3β expression or phosphorylation may contribute to Dex-resistance in CLL cells.

c-IAP2 and *XIAP* are elevated at mRNA level by more than one fold in CLL relative to a panel of lymphoid malignancies (de Graaf, van Krieken et al. 2005) (Section 1.6.7). The IAP family of proteins can suppress apoptosis induced by a range of triggers, and overexpression of XIAP can lead to an accumulation of GC-resistant immature T cells (Conte, Liston et al. 2001). XIAP is reported to possess the most marked anti-caspase activity of the IAP family (Eckelman, Salvesen et al. 2006). Therefore, elevated expression of IAPs, might hinder GC-induced apoptosis in CLL cells.

The aim of this chapter was to elucidate whether pathways important to CLL survival are involved in GC resistance through either constitutive or induced signalling. For the reasons outlined above, NF-κB was the selected as an appropriate start point.

4.2 Materials and Methods

4.2.1 Purification of B lymphocytes

Negative purification of B cells was used rather than positive purification in order to avoid stimulation of the target cells.

2-5x10^7 mononuclear cells from peripheral blood of patients with CLL were incubated in 400µl of 4°C degassed purification buffer (PBS pH7.2, 0.5% BSA, 2mM EDTA) for 20min, in the presence of 20µl of each FITC-conjugated anti-CD3, anti-CD14, and anti-CD16 antibodies (BD Biosciences), which label T cells, monocytes, macrophages, neutrophils, and NK cells. Samples were then centrifuged at 500rcf for 3mins at 4°C, washed in 80µl purification buffer, centrifuged again, and resuspended in 160µl purification buffer. 20µl of anti-FITC magnetic beads (Miltenyi Biotech, Bisley, UK) were then added to each sample.

Cell/magnetic bead suspensions were incubated at 4°C for 15mins and then pelleted, washed, resuspended in 80µl purification buffer, and applied to a Mini Macs column (Miltenyi Biotech) for 5mins. The $CD3^+$, $CD16^+$, or $CD14^+$ cells remain in the column by virtue of the attached beads, which allows collection of the unlabelled CD19+ B cells that flow through.

1ml of buffer was added to the column three times to aid the flow through and collection of B cells. B-cell purity was found to be >93% following this purification, as shown by CD19-PE positivity and CD3, CD14, and CD16-FITC

negativity, relative to IgG stained control samples. A representative CLL case post purification is shown in Figure 4.2.1.

The B cell purity of the CLL samples was determined by flow cytometry, using a Becton Dickinson FACSCalibur machine and a PE-conjugated anti CD19 antibody (Figure 4.2.1). In this example, B cells were shown to account for >97% of the detected events.

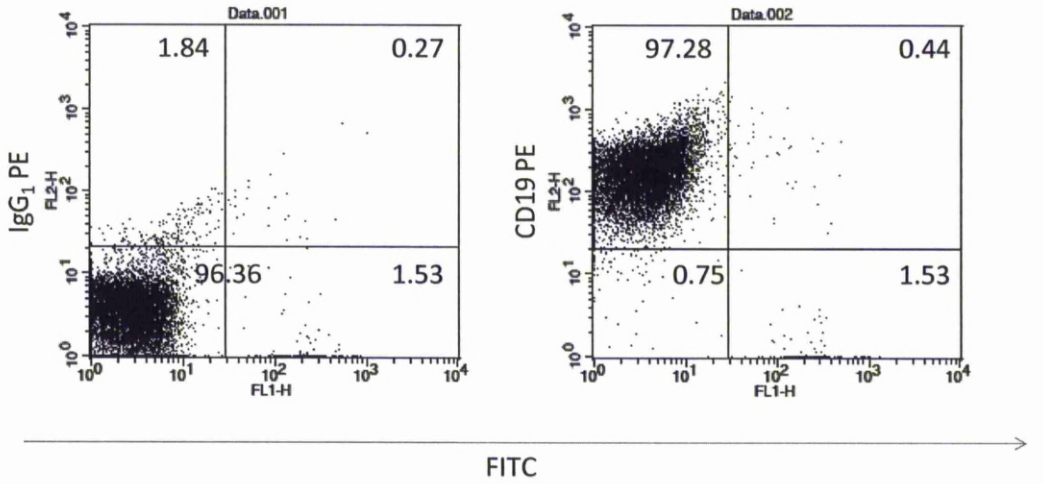


Figure 4.2.1. Representative plots showing B cell purity following purification. CD3, CD14, and CD16-FITC (x-axis) versus IgG-PE (left) or CD19-PE (right) (y-axis). Left. IgG1 acts as a negative control for CD19 binding. Right. Cells are shown to be 97% CD19+/CD3-, CD14-, and CD16- (upper left quadrant). CD3, CD14, and CD16 expressing cell contamination is indicated by FITC positivity (lower right quadrant).

4.2.2 CLL cell culture

Cells were thawed from liquid nitrogen, and cultured at densities of $5x10^6$ cells/ml under the conditions described in Section 2.2.5.

4.2.3 CLL cell stimulation/inhibition

Bay11-7082 (Calbiochem) was diluted in DMSO (dimethyl sulphoxide) and used at a range of concentrations, up to 8μM. CLL cells were incubated at $5x10^6$/ml for 18.5h. DMSO was added to untreated controls as appropriate.

To stimulate cells with CpG/ODN (oligodeoxynucleotides) type-B, CLL cells ($2x10^6$/ml) were cultured in the presence of CpG (Invivogen) at 0.5μg/ml for 4h.

For crosslinking, $F(ab)_2$ fragment of a goat anti-human IgM, $Fc_5\mu$ fragment specific antibody (Jackson ImmunoResearch, Stratech, Soham (UK)) was used to treat CLL cells at 10μg/ml for 10mins at 37°C/5% CO_2.

4.2.4 Standard sample preparation and Western blotting

Samples were lysed either in a clear sample buffer containing 125mM Tris-HCl (pH6.8), 20% glycerol (Sigma), and 4% SDS (Fisher Scientific, Loughborough, Leicestershire, UK), or in RIPA buffer containing 50mM Tris, (pH7.6), 150mM NaCl, 25mM Na-pyrophosphate, 50mM Na-glycerophoshpate decahydrate, 2mM EDTA and 2mM EGTA.

Determination of protein concentration was carried out in duplicate using the Biorad DC protein assay (Biorad, CA, USA), according to the manufacturer's instructions. This assay is similar to the Lowry assay (LOWRY, ROSEBROUGH et al. 1951), in which protein concentration is exhibited by a colour change of the sample solution, involving reduction of the reagent folin and oxidation of

aromatic residues. Samples had to be diluted to protein concentrations of between 0.2 and 1.5 mg/ml to allow reliable quantitation. Protein concentration was measured at 650–750 nm with a standard laboratory spectrophotometer or microplate reader.

The remaining components of SDS-sample buffer were added to samples at final concentrations of 10% β-mercaptoethanol (Sigma) and 0.006% bromophenol blue (VWR International, Lutterworth, Leicestershire, UK) following quantitation and normalisation of sample concentration.

Western blotting was performed as described in Section 2.2.8, using the antibodies specified in Table 4.2.1, below.

Table 4.2.1 Antibodies used in immunoblotting. Antibody incubations were for 1h at R/T or O/N at 4°C.

Primary Antibody	Conditions	Secondary Antibody	Conditions
Calnexin Rabbit PAb Abcam	1/2000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
HDAC1 Mouse MAb MO2 Abnova	1/1000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
Histone H3 Rabbit PAb Call Signalling	1/1000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/2000
Lck Mouse MAb 3A5 Santa Cruz	1/2000 5% Advanced block TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
GSK-3β Rabbit PAb Cell Signalling	1/2000 5% BSA TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
p-GSK-3α/β Rabbit PAb Cell Signalling	1/2000 5% BSA TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
c-IAP2 Rabbit MAb 58C7 Cell Signalling	1/2000 5% BSA TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
XIAP Rabbit MAb 3B6 Cell Signalling	1/1000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
β-Actin Mouse PAb Sigma	1/10000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
p-IκBα Mouse Cell Signalling	1/2000 5% Advanced block TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
IκBα Rabbit PAb Santa Cruz	1/2000 5% Advanced Block TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000

4.2.5 Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed as in Section 3.2.6. Table 4.2.2, below, shows the parameters of the reactions performed.

Table 4.2.2 qRT-PCR conditions

Target	Primer Concentration (pmol/µl)	Master Mix	Anneal Temperature (°C)	Read Temperature (°C)	Product Length (bp)
Bcl-xl	0.2	Dynamo	60	82	309
Bcl-2A1	0.4	Dynamo	58	N/A	539

Primer sequences are shown in the list below.

Bcl-xl

FOR-5'-TGCGTGGAAAGCGTAGACAAG-3'

REV-5'-AGGATGTGGTGGAGCAGAGAA-3'.

Bfl-1

FOR-5'-GGCAGAAGATGACAGACTGTGAA-3'

BAC-5'-TGGTCAACAGTATTGCTTCAGGA-3'

Amplification plots and dissociation curves for PCR are shown if Figure 4.2.2.

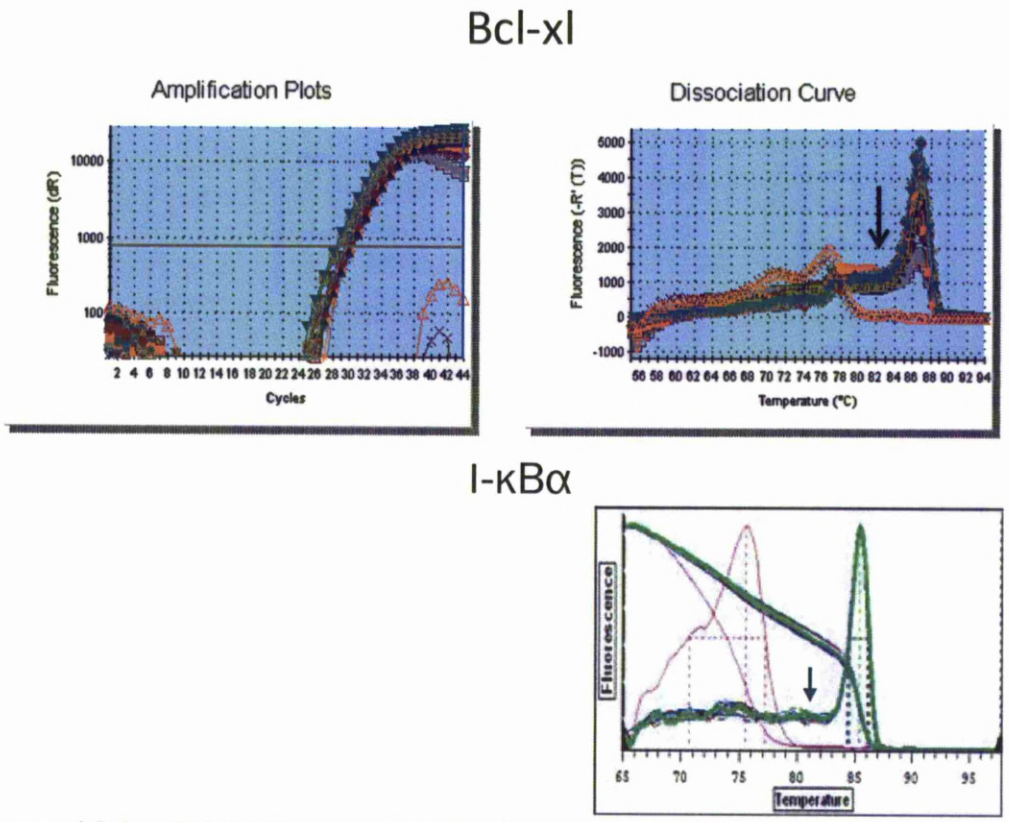


Figure 4.2.2 qRT-PCR amplification plots and dissociation curves. Left. The amplification plot shows placement of the threshold within the exponential phase of amplification for Bcl-xl (horizontal line). Right. The dissociation plots show placement of the read-temperatures for each PCR, beyond interference from primer dimer and any non-specific product (left of blue arrows).

4.2.6 Soluble Nuclear Protein Isolation Optimisation

Non-denaturing nuclear isolation is performed as follows (Jakob 1992): Generally, a hypotonic (of higher osmotic potential than the cells) buffer is used to induce swelling and some disruption of the cytoplasmic membrane. Next, replacement with a hypertonic (of lower osmotic potential than the cells) buffer leads to rapid shrinkage of the cells and is accompanied by extensive cell lysis. Nuclei are then separated from other cellular components by centrifugation. The primary aim is to lyse the cell membrane without lysing the nuclear membrane, which is particularly difficult given that nuclear membrane and cytoplasmic

membranes are structurally and chemically alike. This issue is particularly relevant to CLL cells as they possess particularly large nuclei, which renders their nuclear membranes highly vulnerable to methods of disruption that target the plasma membrane.

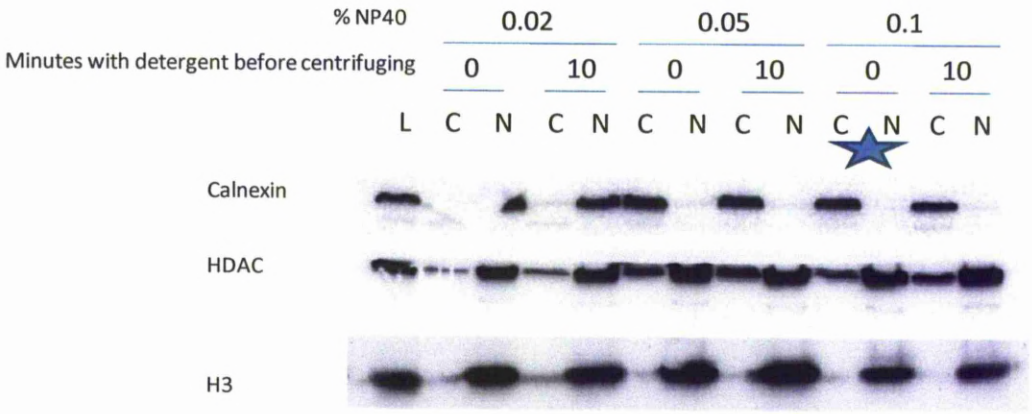
In order to perform functional assays on isolated nuclear proteins, once the nuclear pellet has been isolated, a conservative approach is required to ensure that any active target is maintained in its active state. Therefore, rather than using reagents that alter protein chemistry or structure, a method that makes use of osmotic changes is once again preferable. If the salt concentration is high enough, this approach not only preserves the nuclear membrane so as to keep DNA from engulfing the desired protein, but also strips promoter-bound transcription factors without the need to sheer/digest the DNA. Avoidance of DNA sonication is preferable because it may impact on protein integrity. A further concern is particularly relevant to any NF-κB activity assay. Since migration of this transcription factor from cytoplasm to nucleus is a key event in its activation (De Bosscher, Vanden Berghe et al. 2003), any contamination of the nuclear fraction by the cytoplasmic fraction would invalidate the measurements.

To optimise the nuclear isolation step, a range of hypertonic buffers was tested. These buffers differed in their osmolarity and detergent concentration. Once the basic cellular lysis buffer had been selected, NP40 (detergent) concentration and length of exposure were optimised (Figure 4.2.3). Cells were cultured at $5x10^6$ cells per well of 24-well plates. Simultaneous nuclear protein extractions were performed as described in Section 4.2.7. Samples were lysed in 100µl cell lysis

buffer, and 20µl of sample was loaded per lane (~$1x10^6$ cells worth of material) of a polyacrylamide gel. Western blot for Calnexin (cytoplasmic), HDAC and Histone-H3 (nuclear) (Figure 4.2.3) allowed identification of cytoplasmic and nuclear components, respectively.

The cytoplasmic marker, calnexin, presented mainly in the pelleted nuclear fraction when an NP40 concentration of 0.02% was used, indicating insufficient lysis of the cytoplasmic membrane in the first lysis step. At 0.05% and 0.1% NP40, the cytoplasmic marker almost disappeared from the nuclear fraction, and a 10min incubation with the detergent appeared to have no effect. Therefore, NP40 was required at a minimum concentration of 0.05% in order to achieve adequate lysis of the cytoplasmic membrane. Hence, the optimal conditions were defined as 0.1% NP40 with no incubation between NP40 addition and subsequent centrifugation.

As the Active Motif ELISA method had been selected for subsequent measurement of NF-κB activity, it was decided to use this company's commercially available hypertonic buffer for consistency.



Buffer
10mM HEPES pH7.9
3mM MgCl2
20mM KCL
2mM DTT
Protease and Phosphatase Inhibitors

L = WCL
C=Cytoplasmic
N=Nuclear

Figure 4.2.3 Effect of NP40 concentration and incubation time on segregation of nuclear and cytoplasmic markers following CLL cell nuclear isolation. As expected, Calnexin, HDAC and H3 proteins resolved at 100kDa, 55kDa, and 14kD, respectively. L = WCL, C = cytoplasmic fraction, N = nuclear fraction. The lanes representing the NP40 concentration and detergent incubation time subsequently used in the purification procedure are denoted by a star.

4.2.7 Nuclear Protein Isolation from CLL cells

1/100 DTT (dithiothreotin), 1/100 protease inhibitors, and 1/100 phosphatase inhibitors were added to hypotonic Lysis Buffer A [0.1M $MgCl_2$, 2M Kcl, 0.1M HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)], and to hypertonic TransAM Complete Lysis Buffer (Active Motif, Belgium). Cellular material equivalent to ~$5x10^6$ cells was washed in PBS and resuspended in a small residual amount by vigorous flicking. 180µl Lysis Buffer A was added to samples, which were gently agitated into suspension. Samples were then left on ice for 15min, and 20µl 1% NP40 was added before very gentle agitation. Samples were

immediately spun at 650rcf for 5min. Supernatant was decanted, and samples were washed in a further 200µl Lysis Buffer A. 50µl Complete Lysis Buffer was added, and the nuclear pellet was left on a plate shaker set at 600rpm on ice for 30min, with samples briefly vortexed at high speed every 10min. Samples were centrifuged at maximum speed for 10min, and the supernatant was removed before tubes were transferred to -80°C storage.

Protein determination was performed in duplicate by RC/DC Bradford Assay (Biorad), which overcomes the reducing activity of DTT in Complete Lysis Buffer by precipitation and resuspension of protein samples. The assay was performed in duplicate. Duplicate samples that displayed an average spectrophotometric measurement with CV (percent coefficient of variation) of over 20% were remeasured.

4.2.8 ELISA of NF-κB activity

NF-κB activation status can be measured/inferred by a number of methods. Levels of NF-κB or IκB—the NF-κB suppressor—proteins can be measured by Western blotting. However, I-κB plays a role in inhibiting cytoplsamic NF-κB up-stream in the canonical pathway, and does not indicate differences in NF-κB activity resulting from alternative pathways (Heck, Bender et al. 1997). Furthermore, probing for NF-κB levels within nuclear extracts would not detect differences in activity.

Within *in vitro* systems, reporter genes are considered a highly informative way of measuring transcription factor activity (Nordeen 1988). In studies of NF-κB

activity, luciferase or β-galactosidase can be placed under the control of a promoter that contains an NF-κB consensus binding sequence. Nevertheless, reporter gene assays have to be performed several times to ensure reliability, and assays have to be carefully standardised.

Gel retardation assays, more commonly referred to as Electrophoretic Mobility Shift Assays (EMSA), offer an assessment of DNA binding activity of transcription factors (Fried 1989), such as NF-κB. Cell extracts are incubated with either a radiolabelled or biotin-conjugated double-stranded oligonucleotide probe containing a consensus binding sequence, which only NF-κB that is free from repressors and in its active conformation may bind. Samples are resolved by non-denaturing polyacrylamide gel, followed by autoradiography or streptavidin treatment and chemiluminescent detection of NF-κB-bound probe. This method provides confidence regarding binding specificity as detectable by band resolution, but it requires more cellular material than ELISA-based methods, and is time consuming and inappropriate for high-throughput screening.

Active Motif offers a high-throughput ELISA assay to quantify NF-κB activation by measuring binding of subunits to a consensus promoter sequence. 96-well plates contain immobilised oligonucleotide corresponding to the NF-κB consensus binding sequence (5'-GGGACTTTCC-3'), which the active form of NF-κB specifically binds to. Then, primary antibodies that are specific for promoter-bound epitopes of NF-κB subunits are used to probe. An HRP-conjugated secondary antibody provides a colorimetric readout that is quantified by spectrophotometry. Critically—due to the relatively low yields of nuclear protein

obtained during nuclear purification—this assay is approximately 10-fold more sensitive than EMSA according to the manufacturer.

p65 and p50, and c-Rel are the NF-κB isoforms that warrant particular attention in CLL (Furman, Asgary et al. 2000, Hewamana, Alghazal et al. 2008). p65 homodimers, and p65/p50 heterodimers are transcriptionally active, whilst p50 homodimers are transcriptionally inactive, despite the capacity of the latter to bind NF-κB promoter sequences.

p65 and p50 NF-κB ELISA (TranAM) was performed, according to the manufacturer's instructions (Active Motif, Belgium), to assess the binding capacity of nuclear NF-κB subunits to fixed consensus binding sequence. "Complete Lysis Buffer" (hypertonic lysis buffer B), "Washing Buffer" and "Complete (antibody) Binding Buffer" were prepared first. 30μl of Complete Binding Buffer was added to each well to be used. To demonstrate the specificity of NF-κB subunit binding, each batch of samples included control wells that contained non-fixed competitive NF-κB consensus binding sequence, or a mutated non-competitive version, in excess. For these samples, 30μl of Complete Binding Buffer plus 10pmol of wild-type or mutated consensus oligonucleotide was used. In non-control sample wells, 20μl of sample diluted in Complete Lysis Buffer was added, using between 1 and 20μg of nuclear extract, dependent upon antibody binding efficiency (0.5μg for p50, and 2μg for p65). Positive control wells featured 2.5μg of Jurkat nuclear extract or 5μg of Raji nuclear extract to demonstrate assay validity. Blank wells featured Complete Lysis Buffer alone. Wells were sealed and incubated for 1h at RT with mild agitation via a rocking

platform. Following incubation to facilitate protein binding to fixed oligonucleotide, wells were washed 3 times with 200µl Washing Buffer. p65 or p50 antibodies were diluted 1:1000 and 100µl was added to appropriate wells. The plate was incubated for a further 1h to allow antibody binding to plate-bound NF-κB protein. Wells were then washed x4. Finally, 100µl "Developing Solution" was added to all wells and samples were incubated for between 30s and 5min at RT and protected from direct light until the samples turned from medium to dark blue. 100µl of "Stop Solution" was added and the yellow signals were detected by 450nm spectrophotometry, with a reference wavelength of 655nm. Following data collection, blank wells were subtracted from all readings as background. Positive control/reference samples were used to standardise multiple experiments. The wild-type competitor prevented binding of NF-κB to anchored oligonucleotide, and the mutated version did not, as was expected.

Figure 4.2.4 displays reproducibility between the same lysates tested separately (A), and between duplicate samples of a single assay (B).

p65 ELISA was performed using 12 nuclear protein sample lysates in two separate assays to allow an assessment of assay reproducibility (Figure 4.2.4 A). A strong positive correlation (R^2=0.887, P<0.01) was observed between the two sets of data, which validates the precision of the assay. The histogram (Figure 4.2.4 B) shows ELISA measurements for samples assayed in duplicate within one experiment, with appropriately low standard deviation (+/-0.64-7.69%; mean +/-4.42%).

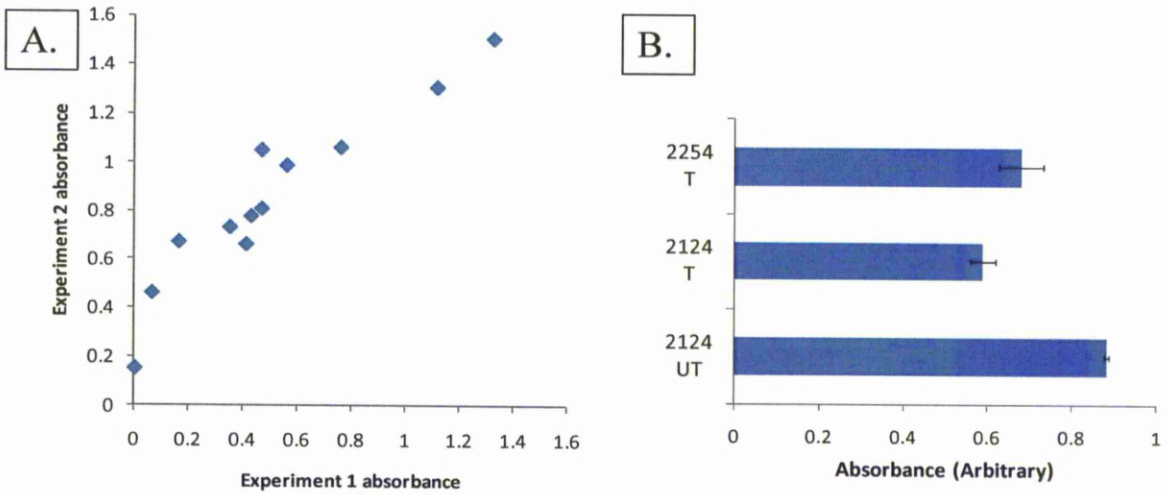


Figure 4.2.4 p65 NF-κB ELISA provides reproducible data. A. ELISA spectrophotometric absorbance for 12 protein samples from two separate assays. R^2=0.887, ***P***<0.01. B. Mean absorbance for duplicate samples of the same assay displaying standard deviation (+/-0.64-7.69%; mean +/-4.42%).

4.3 Results

4.3.1 NF-κB activity

Mutual antagonism between the GR and NF-κB is well substantiated (De Bosscher, Vanden Berghe et al. 2003), and NF-κB is an important mediator of CLL cell survival (Cuni, Perez-Aciego et al. 2004, Hewamana, Alghazal et al. 2008). Furthermore, CLL cells express high constitutive NF-κB activation, and Dex can significantly repress NF-κB activation in CLL samples (Furman, Asgary et al. 2000). Taken together these reports led to the hypotheses that GC resistance in CLL can result from high baseline activation of NF-κB, or from failure of Dex-induced repression of NF-κB activity. p65 and p50 are two of the three most abundant NF-κB isoforms in CLL cells (Furman, Asgary et al. 2000, Hewamana, Alghazal et al. 2008), and the p65/p50 heterodimer is the most common NF-κB dimer in B lymphocytes (Sen 2006). Therefore, ELISA was used to quantify p65/p50 activation status. It was necessary to perform a preliminary timecourse in order to select an appropriate timepoint for comparison of baseline activity and Dex-induced repression. It has already been reported that NF-κB activity can be significantly repressed by 1000nM Dex in CLL (Furman, Asgary et al. 2000) samples by 12h. Therefore, to identify an appropriate timepoint for this study, one sensitive and two resistant samples were cultured for 0, 2, 4, 8, 12, and 16h with and without 100nM Dex, and NF-κB activity was assessed.

Baseline NF-κB activity either increased slightly or remained fairly constant between 0 and 8-12h (Figure 4.3.1). In all samples screened, activity decreased between 12h and 16h. . A clear effect of Dex on NF-κB activity was detectable between 8h and 16h, in keeping with a previous study (Furman, Asgary et al. 2000), at which points Dex reduced NF-κB binding to less than one half of the value in untreated cells at the same timepoint.

Based on the timecourse (Figure 4.3.1), 8h was selected as an appropriate timepoint for further investigation. At this timepoint, the activity of NF-κB in untreated CLL cells was relatively high in all samples, thereby allowing detection of a suppressive effect. 8 sensitive and 8 resistant samples were subsequently cultured for 8h +/- 100nM Dex, and NF-κB promoter binding measured by the ELISA method. The results are shown in Fig 4.3.2.

Consistent with the preliminary results shown in Figure 4.3.1, between 0h and 8h of culture p65 and p50 activation status increased in most samples: 7/8 sensitive samples for p65, and 7/7 sensitive samples for p50; 5/8 resistant samples for p65, and 4/8 resistant samples for p50 (Figure 4.3.2). It is perhaps of note that *in vitro* culture induced NF-κB activation more consistently in the sensitive than resistant samples. More importantly, Dex reduced p65 and p50 activation in sensitive samples, as expected, but also in all but one resistant sample for which data was available (Wilcoxon tests for change: p65 $P<0.000$, p50 $P=0.001$). Given that p50 does not itself interact with the GR, this supports the notion that p50 is commonly associated with p65 in CLL cells.

To properly assess the hypotheses that constitutive or GC-induced NF-κB activity was responsible for GC resistance in this study, the data from Figure 4.3.2 was grouped and contrasted (Figure 4.3.3). Upon statistical analysis, there was no difference between GC-sensitive and -resistant CLL samples in terms of constitutive or Dex-induced p65 activity (Figure 4.3.3 A) ($P>0.05$). Contrastingly, p50 activation levels were lower in resistant samples both in the absence ($P=0.009$) and presence ($P=0.037$) of Dex (Figure 4.3.3 A). Given that p65 activity was clearly repressed more than p50 activity in both groups of samples (4.3.3 B), it is likely that p50 homodimers were detected as well as p65-bound p50. Therefore, p50 might positively contribute to Dex-sensitivity by virtue of transcriptionally inactive homodimers (Sen 2006). However, the median activation levels of p50 in the sensitive group were less than 50% higher than those of the resistant group, calling into question the biological significance of this observation. Moreover, as was the case regards p65 fold change, there was no significant difference in p50 fold change between the two sensitivity groups ($P>0.05$) (Figure 4.3.3 B).

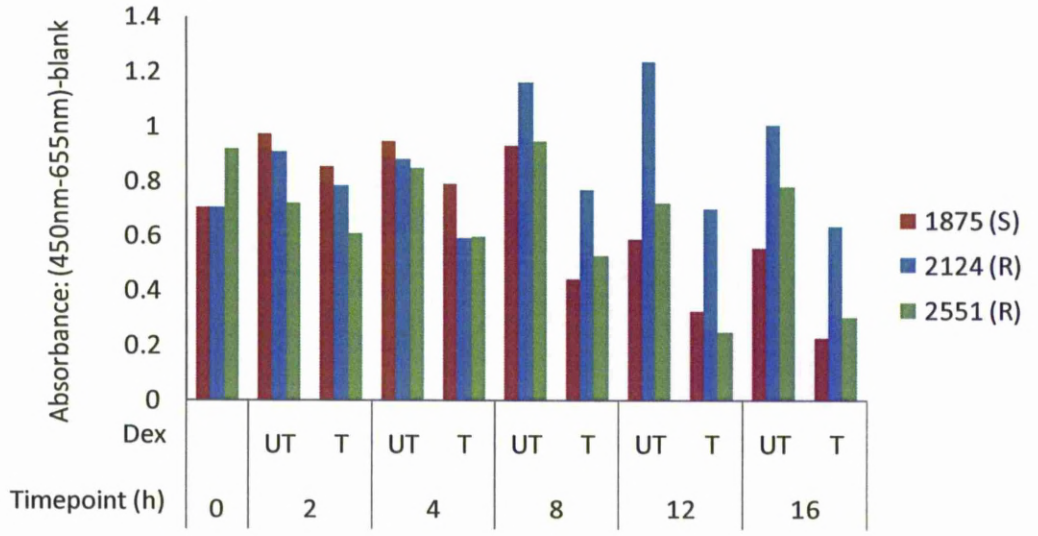


Figure 4.3.1 NF-κB basal activation status and repression by Dex over a 16h timecourse. One sensitive (S) and two resistant (R) samples were cultured for up to 16h w/wo 100nM Dex (UT/T). The resulting samples were used for NF-κB activity screening by promoter binding ELISA, which was measured by spectrophotometry.

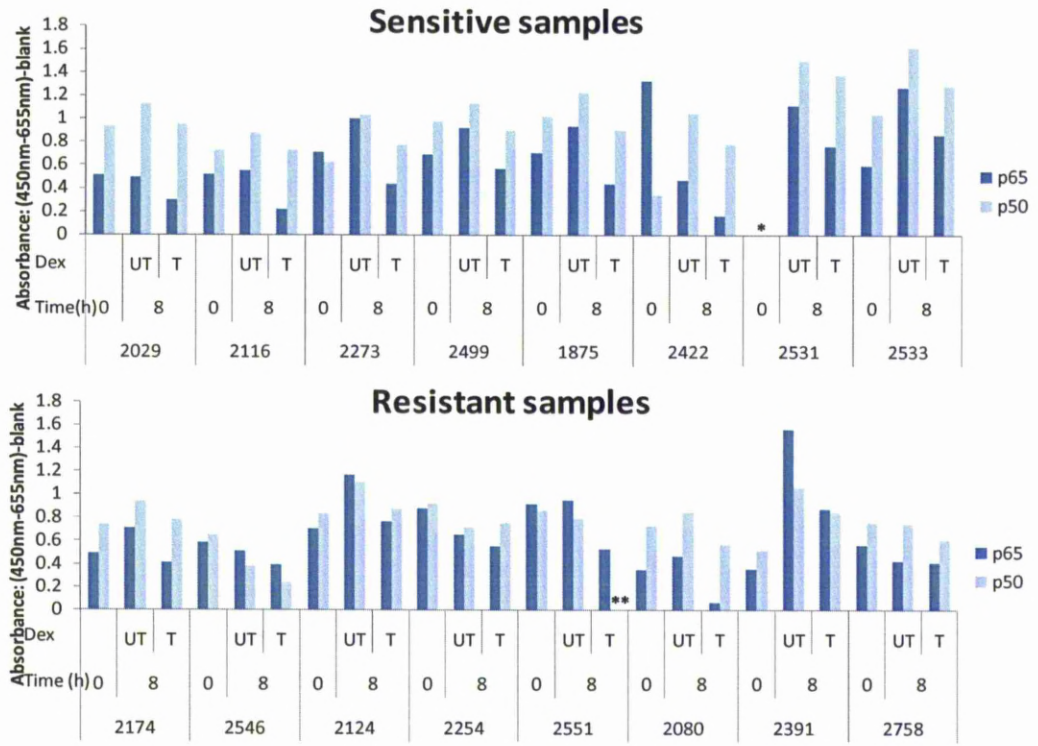


Figure 4.3.2 NF-κB is downregulated by Dex at 8h in both sensitive and resistant samples. *2531 0h p65=0/p50=NO DATA. **2551 T p50=NO DATA. Samples from 8 sensitive and 8 resistant samples were incubated w/wo 100nM Dex for 8h. ELISA of p65 and p50 binding was measured by spectrophotometry. Wilcoxon paired tests for Dex-induced change for 8h samples are as follows: (n=15) p65 $P<0.000$, p50 $P=0.001$.

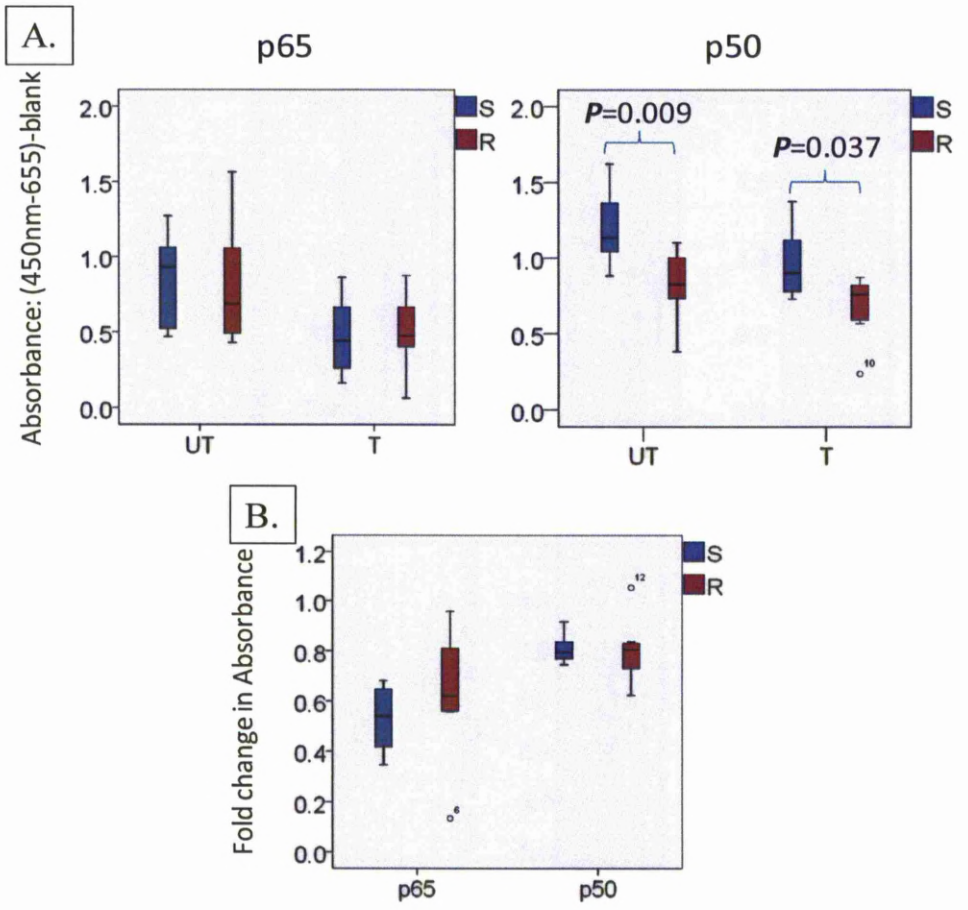


Figure 4.3.3 Stem and leaf plots representing NF-κB activation status in sensitive and resistant samples. A. Stem and leaf plots comparing baseline (UT) and final activation level (T) of p65 and p50. Mann-Whitney *U* tests for group difference are as follows: p65 UT ***P***=0.713 (8S/8R), p65 T ***P***=0.834 (8S/8R); p50 UT ***P***=0.009 (8S/8R), p50 T ***P***=0.037 (8S/7R). B. Fold-change in activation level of p65 and p50. Mann-Whitney *U* tests for group difference are as follows: p65 ***P***=0.294 (8S/8R), p50 ***P***=0.908 (8S/7R).

4.3.2 NF-κB transcriptional activity

There are a number of transcriptional targets of NF-κB the expression of which can impact on cell survival (Horie, Watanabe et al. 2006, Vallabhapurapu, Karin 2009). Bcl-2A1 and Bcl-xl stand out in particular in CLL as having a profound role in survival (Vogler, Butterworth et al. 2009). Whilst IκBα can be induced by NF-κB, it can also be induced by GCs in certain cell types (Auphan, DiDonato et al. 1995, Scheinman, Cogswell et al. 1995), thus providing a further link between GC killing and NF-κB activity. However, GC-mediated induction of IκBα has not been previously reported in CLL. Therefore, in order to gain insight into whether differences in NF-κB pro-survival transactivation existed between sensitive and resistant samples, it was decided to quantify mRNA expression of these three targets. So that the readout was temporally downstream of the NF-κB activation assay screen, samples were cultured w/wo Dex overnight. The results are shown in Figures 4.3.4 and 4.3.5.

CpG oligodeoxynucleotides are short single-stranded synthetic DNA molecules. When these CpG motifs are unmethylated, they act as immunostimulants, which stimulate NF-κB signalling in CLL cells via Toll-like receptors (TLRs) (Muzio, Ni et al. 1997, Muzio, Natoli et al. 1998)(Yi, Peckham et al. 1999). Therefore, CpG stimulated CLL cells can act as positive controls for detection of NF-κB-induced factors. CpG treatment clearly induces a band of the appropriate size for *Bcl-2A1* and also augments the expression of a band that is consistent with *Bcl-xl* (Figure 4.3.4), which validates the PCR conditions for these targets. *ACTB* detection in all samples indicates acceptable sample quality and concentration. *Bcl-xl* was detectable in CLL cells that were cultured overnight as well as in cells

that were cultured for 4h. However, *Bcl-2A1* was not detected in the overnight samples at sufficient levels to allow quantitation, and was only faintly detectable at 4h without CpG stimulation. *Bcl-xl* and *IκBα* alone were therefore used as measures of NF-κB activity in order to further explore the hypotheses that GC resistance results from either high baseline NF-κB activity or failure of GC-induced repression of NF-κB activity.

Neither *IκBα* nor *Bcl-xl* mRNA expression was consistently altered by Dex ($P>0.05$) (Figure 4.3.5). Furthermore, expression of both transcripts at baseline and Dex-induced fold change was not significantly different between the two groups ($P>0.05$). A difference did exist between the two sensitivity groups in *Bcl-xl* expression of Dex-treated samples, as the sensitive group displayed close to double the median expression level of the resistant group. However, this finding was counterintuitive, as Bcl-xl is upregulated by NF-κB and is a pro-survival member of the Bcl-2 family of proteins. It is plausible that this difference was biologically negligible, as there appears to be considerably less *Bcl-xl* mRNA at 21.5h than 4h (Figure 4.3.4).

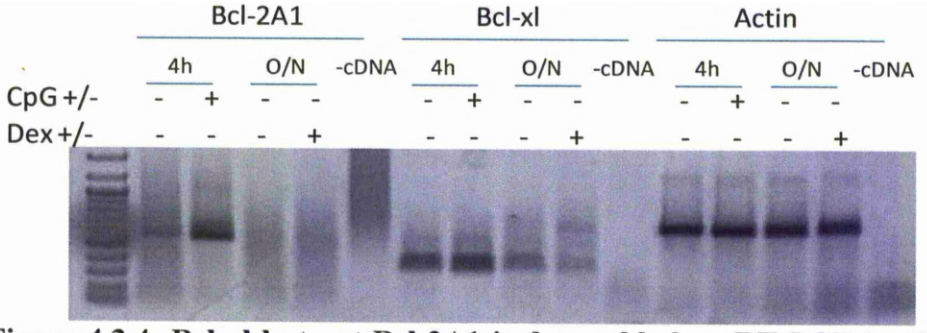


Figure 4.3.4 Bcl-xl but not Bcl-2A1 is detectable by qRT-PCR in CLL cells cultured overnight. qRT-PCR was performed to amplify *Bcl-xl* (309bp) and *Bcl-2A1* (539bp) in a CLL sample cultured w/wo CpG treatment for 4h, and in a CLL sample cultured w/wo 100nM Dex for 21.5h. *ACTB* (Actin) (626bp) was detected to estimate sample quantity/quality. Products were separated and visualised by AGE.

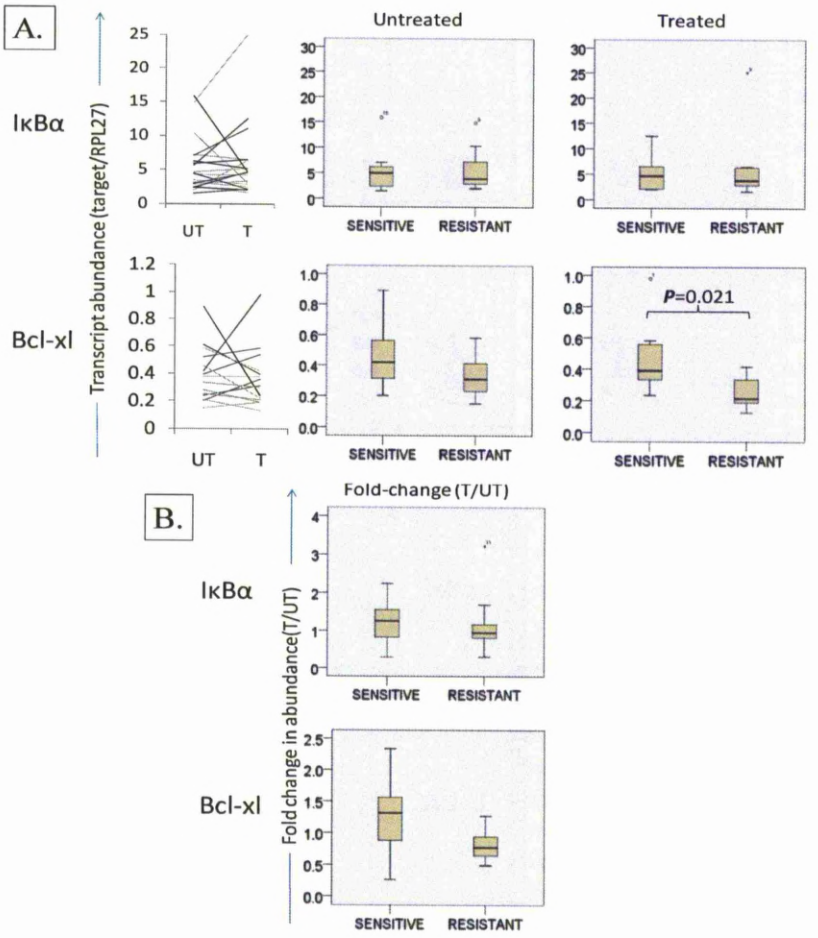


Figure 4.3.5. *IκBα* and *Bcl-xl* mRNA expression is not associated with sensitivity group. 10 sensitive (black lines) and 10 resistant samples (grey lines) were screened for *Bcl-xl* and *IκBα* expression by qRT-PCR after being cultured for 21.5h w/wo 100nM Dex. A. Relative transcript abundance w/wo Dex. *IκBα* levels do not change in a consistent manner (***P***=0.794, Wilcoxon test). *Bcl-xl* levels do not change in a consistent manner (***P***=0.460, Wilcoxon test). GC-sensitive and -resistant samples are not significantly different in terms of (A) *IκBα* baseline and final expression, or (B) fold-change (***P***=0.940 (UT), ***P***= 0.762 (T), ***P***=0.545 (FC), Mann-Whitney *U* test). GC-sensitive and –resistant samples are not significantly different in terms of *Bcl-xl* baseline expression (A) or fold-change (B), but are with respect to (A) expression following Dex treatment (***P*** = 0.247 (UT), 0.021 (T), 0.132 (FC)).

4.3.3 NF-κB inhibition

The canonical pathway of NF-κB activation is controlled by IκBα and upstream kinases (Neumann, Naumann 2007). Active, unphosphorylated, IκBα antagonises p65 and p50, which are predominantly activated by the canonical pathway (Sen 2006). To further test the idea that NF-κB pro-survival activity may have a central role in Dex resistance, Bay117082, an inhibitor of IκBα degradation-targeting phosphorylation (Pickering, de Mel et al. 2007), was added to 3 resistant samples w/wo Dex for overnight culture. A range of concentrations of NF-κB inhibitor was used to span the known lethal dose that has been previously used within this research group. Similarly, the timepoint for this study was based upon the known efficacy of Bay117082. The results are shown in Fig 4.3.6. p-IκBα and, to a lesser extent, IκBα itself, were downregulated by the higher doses (4 and 8μM) of Bay117082 in all three resistant samples at 17.5h (Figure 4.3.6 A), as expected (Pickering, de Mel et al. 2007). However, whilst Bay117082 alone displayed dose-dependent cytotoxicity it did not sensitise CLL cells to killing by Dex at any concentration (Figure 4.3.6 B). Taken together, the various data measuring NF-κB activity does not support a critical role for NF-κB in resistance of CLL samples to GC-induced killing.

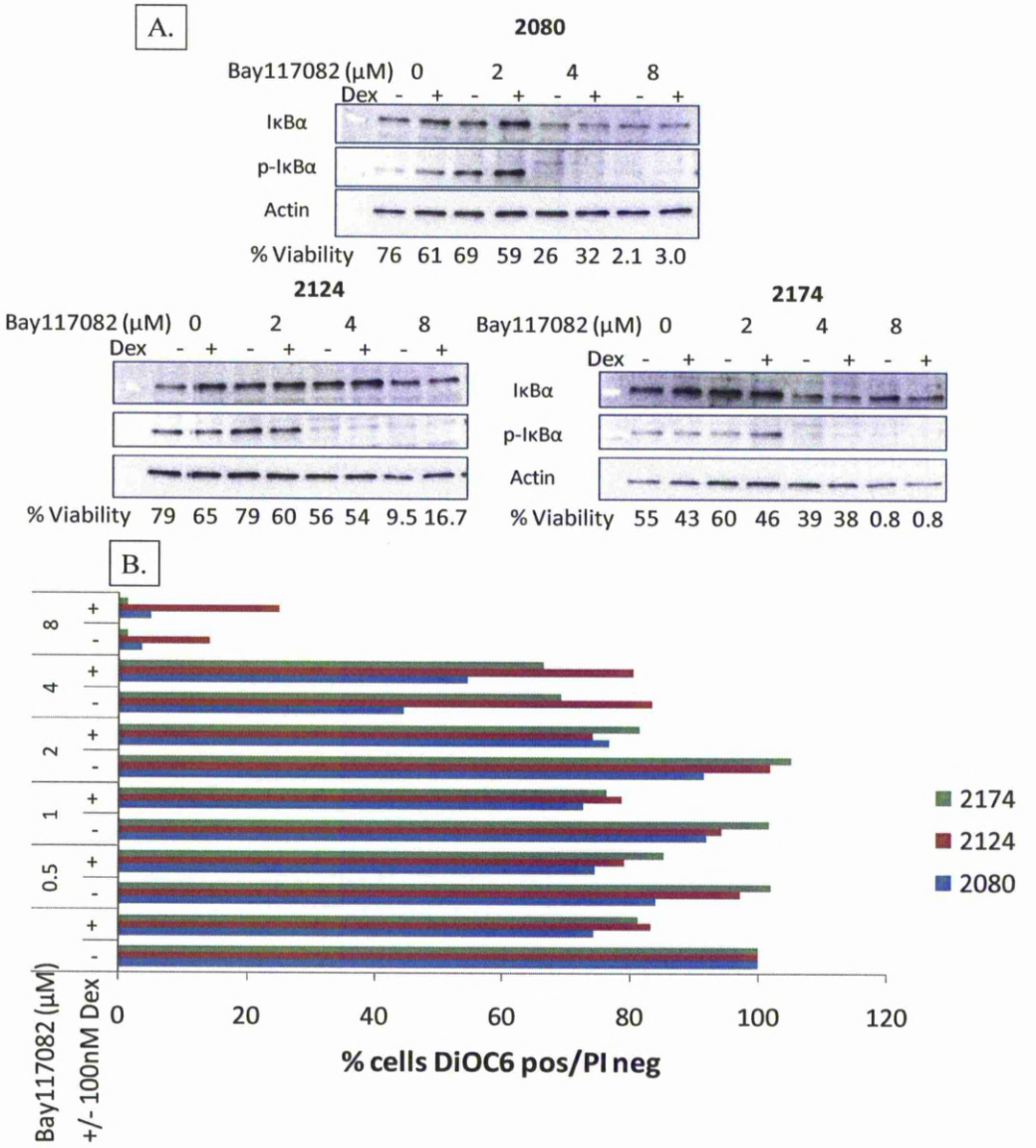


Figure 4.3.6. Inhibition of the canonical NF-κB pathway does not facilitate killing of CLL cells by Dex. Three Dex-resistant CLL samples were incubated w/wo 100nM Dex overnight with a range of concentrations of Bay117082. Viability was determined by $DiOC_6$/PI staining. A. Western blots for IκBα/p-IκBα, and Actin. B. Relative viability data as determined by $DiOC_6$/PI.

4.3.4 Lck and GSK-3β

Unpublished data from this department indicates a prominent role for Lck in BCR signalling in CLL cells. Furthermore, a negative correlation between *Lck* mRNA expression and Dex-sensitivity has been reported in CLL samples (Harr, Caimi et al. 2010). GSK-3β is a BCR/PI3-K-regulated mediator of cell survival. When phosphorylated on Ser_9 the kinase possesses prosurvival activity . GSK-3β is also strongly implicated in GC resistance and inhibition of Bim-induced killing in ALL via the PI3-K/Akt survival pathway (Spokoini, Kfir-Erenfeld et al. 2010). In light of these considerations, it seemed plausible that Dex-induced killing might be influenced by baseline expression of Lck and GSK3-β and the Ser_9 phosphorylation status of GSK3-β. Therefore, GC-sensitive and –resistant CLL samples were compared for baseline expression of Lck, total GSK3-β and p-GSK-3β (Ser_9). The results are shown in Fig 4.3.7.

As T cells highly express Lck, B cells were purified from 6 sensitive and 6 resistant samples prior to this kinase screen. A Jurkat T-cell line sample and a cross-linked CLL sample (BCR-simulated) served as positive controls for Lck, and p-GSK-3β. No difference was observed between the two groups in terms of baseline expression of these proteins or GSK-3β Ser_9 phosphorylation. This suggests that baseline Lck expression and baseline GSK-3β expression/activation do not strongly influence GC sensitivity in CLL cells.

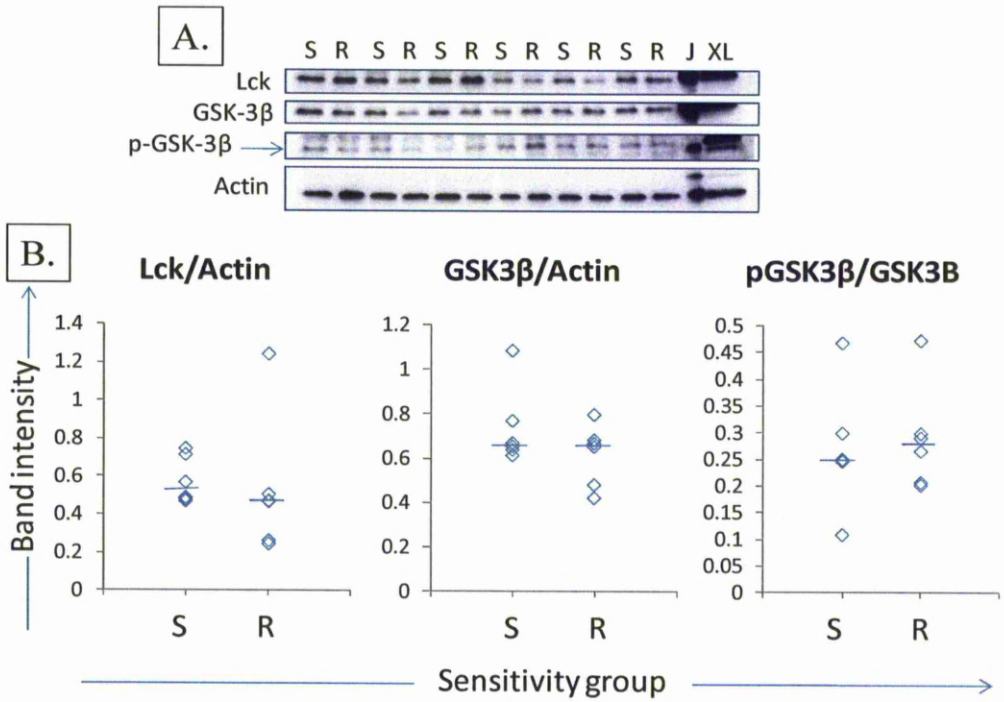


Figure 4.3.7 Baseline expression of Lck, GSK-3β, and GSK-3β does not correlate with GC-sensitivity group. B cells were purified from 6 samples from each sensitivity group. A. Expression of Lck (56kDa), GSK-3β (46kDa), and p-GSK-3β was measured by Western blot using (10μg) RIPA lysates. Actin (40kDa) was used as a loading control. "S" = sensitive sample; "R" = resistant sample; J = Jurkat; XL = a cross-linked CLL sample. B. Densitometry for "A". Horizontal lines represent median expression for each group; S = sensitive samples, R = resistant samples. Mann-Whitney *U* tests for group difference are as follows: Lck ***P***=0.2; GSK-3β ***P***=0.522; p-GSK-3β ***P*** =0.749.

4.3.5 XIAP and c-IAP2 expression

Despite the evidence that GCs are not absolutely dependent on caspases to kill lymphoid cells (Kroemer, Martin 2005), overexpression in resistant samples before or after treatment could slow down killing and contribute to a resistant phenotype. The IAPs are also reported transcriptional targets of NF-κB (Horie, Watanabe et al. 2006) (Section 4.2.1). IAPs have a well-reported role in apoptotic suppression (LaCasse, Mahoney et al. 2008) and XIAP has previously been linked to GC resistance in T cells (Conte, Liston et al. 2001). Baseline levels of c-IAP2 and XIAP (c-IAP1 proved difficult to quantify) were measured to test the hypothesis that GC resistance results from increased expression of these IAPs. XIAP levels were measured before and after treatment with Dex in order to test the idea that Dex resistance might be related to defective repression of XIAP expression. Upon analysis of densitometry data produced by Western blot, neither baseline nor induced IAP expression correlated with sensitivity group (All $P>0.05$) (Figure 4.3.8). Therefore, it would not appear that GC resistsance in this investigation is the result of elevated IAP expression.

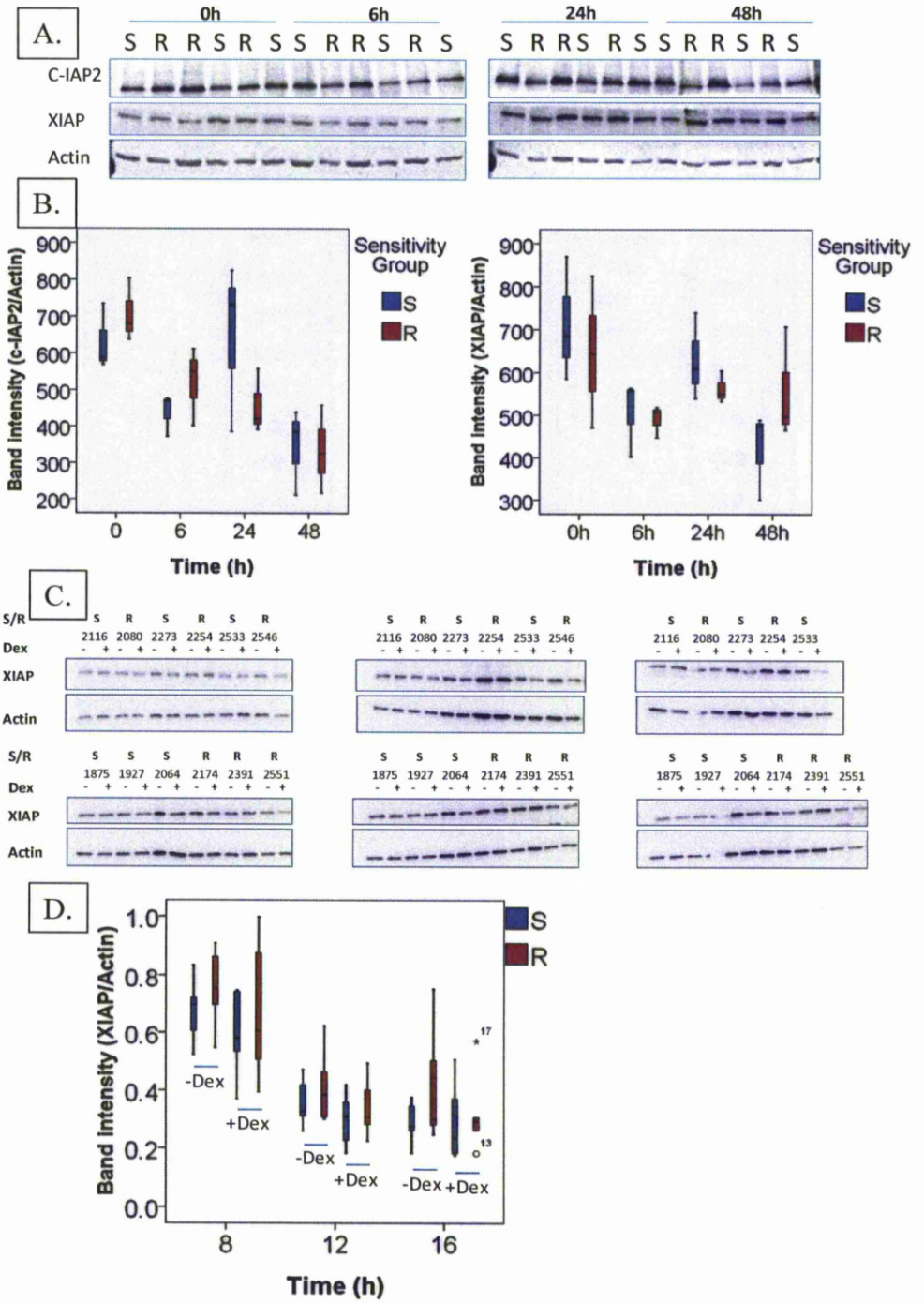


Figure 4.3.8. XIAP and c-IAP2 protein expression does not correlate with GC-sensitivity group. A. c-IAP2 (68kDa) and XIAP (53kDa) Western blots of untreated samples (5μg) from 3 sensitive and 3 resistant samples at 0, 6, 24, and 48h. Actin (42kDa) was used as a loading control. B. Stem-leaf plots for group comparisons of band intensity from "A". Mann-Whitney *U* tests for group difference are as follows: c-IAP2 ***P***=0.275 (0h), ***P***=0.275 (6h), ***P***=0.513 (24h), ***P***=0.827 (48h); XIAP ***P***=0.513 (0h), ***P***=0.513 (6h), ***P***=0.275 (24h), ***P***=0.275 (48h). C. Western blots (10μg per sample) of XIAP expression at 8h, 12h, and 16h in sensitive and resistant samples +/- 100nM Dex. D. Stem and leaf plots of band intensity from "C". Mann-Whitney *U* tests for group difference are as follows: XIAP -Dex ***P***= 0.2 (8h), ***P***= 0.631 (12h), ***P***=0.465 (16h); XIAP +Dex ***P***=0.749 (8h), ***P***=0.873 (12h), *P*=0.361 (16h).

4.4 Discussion

The purpose of this chapter was to identify whether NF-κB signalling—a key CLL survival pathway (Cuni, Perez-Aciego et al. 2004) and source of cross-talk with GR signalling (De Bosscher, Haegeman 2009)—was an important mediator of GC resistance in CLL. Following the rejection of the hypotheses relating to this question, the chapter was expanded to include the kinases Lck and GSK-3β, as well as IAP proteins.

p65 baseline activation at 8h was found to be similar in both groups. However, p50 baseline expression appeared to be elevated in the sensitive samples. Nevertheless, the median activation level in the sensitive group was less than 50% higher than those of the resistant group, and it is therefore unlikely that this feature plays a critical role in GC resistance. Furthermore, there was no observed difference in fold-change in NF-κB activity upon Dex treatment.

ELISA of nuclear NF-κB promoter-binding capacity cannot detect all plausible alterations in NF-κB activity, as it only measures whether NF-κB is both within the nucleus and possesses the ability to bind to a consensus promoter sequence. The use of a consensus binding sequence does not take into account promoter context, enhancer sequences, and chromatin structure. Furthermore, competition between the GR and NF-κB for shared promoters; competition for coactivators; inhibitory tethering of NF-κB by the GR, and co-association related post-translational modifications of NF-κB have not been addressed within this study. Such variables could be assessed by chromatin immunoprecipitation (promoter-binding competition), standard immunoprecipitation (GR tethering of NF-κB,

coactivator binding), and Western blotting (NF-κB modifications). Nevertheless, nuclear localisation, promoter-binding site blocking of NF-κB by GR, and promoter binding related post-translational modification of NF-κB would be detected by the type of assay that was used in this study. Most importantly, Dex treatment of CLL has already been reported to substantially downregulate NF-κB activity as detected by EMSA (Furman, Asgary et al. 2000), which provides similar insight to ELISA. Therefore, the assay used here can be deemed relevant despite the many models for explaining GR and NF-κB cross talk (De Bosscher, Vanden Berghe et al. 2003, Nissen, Yamamoto 2000).

The relatively early timepoint of 8h was used so that high basal activity of NF-κB could be contrasted with the activity measured in Dex-treated samples. Though this timepoint was given validity by a previous study that focused on 12h (Furman, Asgary et al. 2000), it may be worth expanding this study in future to include timepoints closer to the 48h timepoint selected for sensitivity group designation.

Bcl-xl and *IκBα* were used as readouts of NF-κB activity. Whilst *Bcl-2A1* proved difficult to detect in CLL cells from overnight culture, *Bcl-xl* and *IκBα* were quantifiable. Neither a consistent change nor a difference between the two groups in terms of baseline or fold-change mRNA abundance was detected. Counterintuitively, prosurvival *Bcl-xl* expression following treatment was higher in the sensitive group. However, as *Bcl-xl* expression appeared to be lower at baseline at 21.5h—the timepoint used for quantitation—than 4h, the significance of changes in expression at the former timepoint is weakened. Whilst many other

potential transcriptional targets exist that could have been used to measure NF-κB prosurvival signalling (Horie, Watanabe et al. 2006, Vallabhapurapu, Karin 2009), it was practical to only quantify a small number of targets. Again, timepoints for screening could be expanded, but the single overnight timepoint was considered reasonable based on its temporal positioning between the NF-κB activity screen (8h) and the timepoint selected for sensitivity designation (48h).

The extent of Dex-induced killing was not increased at sublethal or lethal doses of Bay117082. Though a single timepoint was used, and this was different from the timepoint used for sensitivity designation, the $DiOC_6$/PI killing assay provides higher sensitivity than using PI alone. Other NF-κB inhibitors do exist, but given the fact that p65 and p50 are controlled predominantly by the canonical pathway of NF-κB activation (Sen 2006), Bay117082 seemed sufficient. Taken together, the NF-κB-related data, along with the *GILZ* mRNA expression data from Section 3.3.3, argue against a critical role for NF-κB in GC resistance in CLL.

Baseline (t0) Lck and GSK-3β expression and Ser_9 phosphorylation did not correlate with sensitivity group. This pilot study used 6 sensitive and 6 resistant samples. Lck phosphorylation was not measured as part of this pilot screen due to time constraints, though this variable may be worthy of future characterisation due to its importance in Lck-mediated signalling (Harr, Caimi et al. 2010). Furthermore, an important role for GSK-3α has been reported in ALL (Spokoini, Kfir-Erenfeld et al. 2010), and so it may be worth studying this protein in CLL within the context of GC signalling. It may also be worth expanding this initial

study in the future to identify whether GCs alter Lck or GSK-3 activity in CLL cells.

It was decided to identify whether baseline or Dex-induced expression of IAP proteins correlated with sensitivity group. Both hypotheses were rejected convincingly with respect to XIAP, as samples were from a number of timepoints. However, c-IAP1 was not included in this screen as it was difficult to detect a high resolution band with the available antibody, and the effect of Dex on c-IAP2 was not investigated. Nonetheless, XIAP is the member of this family with a reported link to GC resistance in lymphoid cells (Conte, Liston et al. 2001).

Rather than expand into other areas of GR crosstalk, of which there are many (Section 1.6), it was decided to perform studies downstream in the killing pathway model to study the machinery of killing (Section 1.7).

Chapter 5: A central role for Bim in GC-induced killing of CLL cells

5.1 Introduction

In the previous chapter it was demonstrated that NF-κB signalling is not fundamental to GC resistance in this study. To a lesser extent, Lck, and GSK-3β involvement in GC resistance was not supported. Additionally, further downstream, a contribution of IAPs to GC resistance in these samples was not found. Although an argument could be made to investigate other signalling molecules that could impact on GR function (Section 1.6), it was instead decided to consider the possibility that GC resistance might result from blockade of the mitochondrial death pathway (Section 1.7.1). This approach was supported by the observations of Section 2.3 that confirmed the involvement of apoptosis, which was clearly more demonstrable in sensitive samples.

Apoptosis plays a critical role in the killing of malignant cells by chemotherapy and radiation, particularly in lymphoid cells (Johnstone, Ruefli et al. 2002, Cory, Adams 2002) (Section 1.4.3). The primary model of intrinsic apoptosis activation depends upon proapoptotic members of the family binding and antagonising antiapoptotic members, which leads to indirect activation of the effectors, Bax and/or Bak (Danial, Korsmeyer 2004). Alternatively, an indirect activation model has been reported, whereby Bax and Bak are bound and activated directly by proapoptotic proteins (Kuwana, Bouchier-Hayes et al. 2005). Following Bax/Bak activation, pores are formed in the outer mitochondrial membrane resulting in

mitochondrial depolarisation and release of cytochrome c and Smac/DIABLO, which activate caspases (Danial, Korsmeyer 2004).

In CLL cells the expression of the *BCL-2* gene family is shifted towards protection from apoptosis (Gottardi, Alfarano et al. 1996), and Bcl-2 members are frequently overexpressed in leukaemic cells (Ploner, Schmidt et al. 2005) (Section 1.7.2). Furthermore, induction of proapoptotic BH3 proteins (Wang, Malone et al. 2003a, Han, Flemington et al. 2001) and repression of their antiapoptotic counterparts (Chauhan, Auclair et al. 2002, Casale, Addeo et al. 2003) has been observed in response to GCs (Section 1.7.3).

Studies of a panel of B-ALL cell lines have previously shown Bim protein induction to correlate with GC sensitivity, and siRNA knockdown of Bim expression was related to increased cell survival (Abrams, Robertson et al. 2004). *Bim* mRNA was upregulated by Dex in both sensitive and resistant samples following GC treatment in Section 3.3.3. Given the importance of Bim in the GC-induced killing of multiple cell types (Bachmann, Gorman et al. 2005, Lu, Quearry et al. 2006, Bouillet, Metcalf et al. 1999, Abrams, Robertson et al. 2004, Erlacher, Michalak et al. 2005, Lopez-Royuela, Balsas et al. 2010), and since Bim expression can be regulated at the post-transcriptional level, it was decided to begin this investigation by comparing Bim protein upregulation in GC-sensitive and –resistant CLL samples. As described in Section 1.7.4, the three major isoforms—Bim-EL, Bim-L, and Bim-S (Figure 1.6)—are consistently observed in haematopoietic cells, including CLL (Kfir-Erenfeld, Sionov et al. 2010).

5.2 Materials and Methods

5.2.1 CLL cell and PBMC culture

Cells were thawed, and incubated at 2-5x10^6 cells/ml in RPMI supplemented with 10% FCS (Sigma), 2mM L-Glutamine (Invitrogen), 100U/ml penicillin, and 100μg/ml streptomycin (Invitrogen), as described in Section 2.2.4.

5.2.2 Inhibitor studies

In some experiments, CLL cells (2x10^6/ml/well) were incubated with the MEK inhibitor—U0126 (Calbiochem)—at a final concentration of 10μM . In other experiments, Z-VAD (OMe) FMK Caspase Inhibitor I (Calbiochem) was used at a final concentration of 50μM after preincubation of CLL cells (5x10^6/ml/well) for 1h under standard culture conditions. Both of these inhibitors were diluted in DMSO, which was added to untreated control samples at concentrations equivalent to those of inhibitor treated samples.

5.2.3 Coculture of CLL cells with CD154 expressing cells

Parental and hCD154-overexpressing NIH 3T3 mouse fibroblast cell lines were provided by Prof. G Cohen at the MRC Toxicological Unit, Leicester. Cells were subcultured in high-glucose DMEM with 10% heat-inactivated FCS, 2mML-glutamine, and 1% penicillin/streptomycin, and kept in culture until passage 25-30. CLL cells were cocultured with the monolayer cells at 3x10^6 cells in 1ml of RPMI, containing IL-4 at a final concentration of 10ng/ml.

5.2.4 Cell lines

Raji cells (human Burkitt Lymphoma cell line) were maintained in RPMI supplemented with 10% Fetal Calf Serum (FCS; Biosera, Ringmer, East Sussex, UK), 2mM L-Glutamine, 100U/ml penicillin, and 100mg/ml streptomycin. The cells were cultured at 37°C in the presence of 5% CO_2.

Jurkat cells (human acute T-cell Leukaemia cell line) were maintained in RPMI supplemented with 10% Fetal Calf Serum (FCS; Biosera, Ringmer, East Sussex, UK), 2mM L-Glutamine, 100U/ml penicillin, and 100mg/ml streptomycin. They were cultured at 37°C, in the presence of 5% CO_2.

HeLa cells (human epithelial carcinoma cell line) were maintained in Eagle's medium supplemented with 10% Fetal Calf Serum (FCS; Biosera, Ringmer, East Sussex, UK), 2mM L-Glutamine, 100U/ml penicillin, and 100mg/ml streptomycin. The cells were cultured at 37°C, in the presence of 5% CO_2.

5.2.5 Sample preparation and standard Western blotting

Following incubation for 0, 12, and 24h w/wo 100nM Dex, cells from 6 sensitive and 6 resistant samples were washed in PBS, then lysed in 200µL SDS Sample Buffer containing 125mM Tris-HCl (pH6.8), 20% glycerol (Sigma), 4% SDS (Fisher Scientific, Loughborough, Leicestershire, UK), 10% β-mercaptoethanol (Sigma) and 0.006% bromophenol blue (VWR International, Lutterworth, Leicestershire, UK). Samples were then sonicated at 30Hz for 4 x 5secs, and incubated at 95°C for 5mins.

Separate aliquots of cells were lysed in the same SDS Sample Buffer except in the absence of reducing agent and dye, to allow normalisation of protein concentration prior to Western blotting. β-mercaptoethanol and bromophenol blue were subsequently added to samples prior to Western blotting.

Further samples were lysed in RIPA (RadioImmunoPrecipitation Assay) buffer, containing 50mM Tris, (pH7.6), 150mM NaCl, 25mM Na-pyrophosphate, 50mM Na-glycerophoshpate decahydrate, 2mM EDTA, and 2mM EGTA.

Protein determination was carried out in duplicate using the Biorad DC protein assay (Biorad, CA, USA) according to the manufacturer's instructions. Following protein concentration normalisation, remaining components of SDS-sample buffer were added to final concentrations of 10% β-mercaptoethanol (Sigma) and 0.006% bromophenol blue (VWR International, Lutterworth, Leicestershire, UK).

Either 20µl of 200µl of total sample, or between 5µg and 20µg of total protein per well were loaded to lanes in Western blotting. Western blotting was performed as described in Section 2.2.8, using the conditions described in Table 5.2.1.

5.2.6 Cell viability by PI/FACS

40µl aliquots of resuspended cells were added to 80µl of PI in PBS at 5µg/ml for 30mins on ice, before analysis as described in Section 2.2.6.

Table5.2.1 Antibodies used in immunoblotting. Antibody incubations were for 1h at R/T or O/N at 4°C.

Primary Antibody	Dilution	Secondary Antibody	Dilution
Bim Rabbit PAb Cell Signalling	1/2000 5% BSA TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
Actin Mouse MAb AC-74 Sigma	1/10,000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
p-ERK 1/2 Mouse MAb E-4 Santa Cruz	1/2000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
ERK 1/2 Rabbit PAb Santa Cruz	1/10,000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
Bcl-2 Rabbit PAb Santa Cruz	1/1000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/2000
Tubulin Mouse PAb Abcam	1/10,000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
Bax Mouse MAb 3 BD Pharmingen	1/2000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
Bak Rabbit PAb Santa Cruz	1/2000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000
Bcl-2 Mouse MAb 100/D5 Abcam	1/500 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/2000
Mcl-1 Mouse MAb 22 BD Pharmingen	1/1000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/2000
PARP Mouse MAb C2-10 RnD Systems	1/4000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/2000
Bcl-xl Rabbit PAb BD Pharmingen	1/1000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/2000
DLC8 Mouse MAb EP1660Y Abcam	1/2000 5% milk TBS-T	Goat Anti-Mouse HRP Santa Cruz	1/5000
Mcl-1 Rabbit PAb Santa Cruz	1/2000 5% milk TBS-T	Goat Anti-Rabbit HRP Santa Cruz	1/5000

5.2.7 Assessment of cell viability by $DiOC_6$ and PI dual staining

Aliquots of 40µl cells were taken and added to 40µl $DiOC_6$ for 15mins at 37°C/5% CO_2. Then, samples were added to 160µl PI (5µg/ml) in PBS and incubated for 30mins on ice before analysing as described in Section 2.2.7.

5.2.8 Bax/Bak Immunoprecipitation

Bax IP lysis buffer contained 10mM HEPES (pH7.4), 150mM NaCl, 2mM EDTA, 1% 3-[(3-chlolamidopropyl)dimethylammonio]-1propanesulfonatehydrate (CHAPS) (Sigma, UK), and 1/100 protease and phosphatase inhibitor cocktails. A buffer of the same composition but with 0.2% CHAPS was used for washing steps.

Samples were first washed in ice-cold PBS before adding 200µl of lysis buffer. They were then agitated on a rotor mixer at 4°C for 1-2h. Sepharose A or G beads were pre-rinsed in lysis buffer. Samples were centrifuged at 13,000rcf for 15secs at 4°C, and pellets were discarded. 30µl of both Sepharose A and G beads were added to each sample, which were then rotor-mixed for 1h at 4°C (preclearing). Samples were pulse centrifuged at 10,000rcf, and protein concentration of the pre-cleared supernatant was measured by DC protein assay (Biorad), as previously described. 100µg of protein/sample was added to 2µg/1µg of Bax IgG_1 6A7 (Sigma) or Bak IgG_2A Ab-1 (Calbiochem) antibody, respectively. Samples were incubated with 30µl beads per sample for 1h on a rotor mixer, and then pulse centrifuged again. Beads were washed in IP wash buffer and then resuspended to a final volume of 30µl total SDS sample buffer.

5.2.9 Bim Immunoprecipitation

300µl RIPA (containing 1/100 phosphatase inhibitor) was added to each sample prior to sonication. Protein A beads were prewashed and 50µl slurry was used to preclear each sample for 1h at 4°C on a rotor mixer. The beads were discarded

after the samples had been carefully removed. For IP, 100µg sample was made up to 100µl, and 1µg Bim Rabbit PAb (Cell Signalling) was added. In the case of IgG controls, 1µg IgG-Rabbit antibody was added. Samples were mixed overnight on a rotor mixer at 4°C. The following day, Protein-A beads were pre-rinsed and 30µl slurry was added to each sample. Samples were mixed for between 1 and 2h at 4°C on the rotor mixer. The supernatant was very carefully removed and stored after high-speed centrifugation. Beads were washed twice in 400µl RIPA buffer, and were made up to 40µl prior to addition of 6x Loading Buffer. Samples were then briefly vortexed and heated at 95°C for 10mins.

5.2.10 Bim-IP Western blots

Large 15% polyacrylamide gels with 5% stacking layers were used to separate out IP protein bands. 10µg of RIPA whole-cell lysate was loaded for each sample (10% of protein used for Bim IP), along with the entire IP samples and 10% of their equivalent supernatants post-IP. The gels were run over 24h at between 50V and 300V until the dye front had migrated to the very edge of the gels. Blots were transferred to Immobilon PVDF membrane for 2h at 400mA, and were then blocked in 5% Advanced Blocking Reagent for 30m. Exactacruz (Santa Cruz) secondary antibodies were used rather than standard antibodies to reduce heavy and light-chain background.

5.2.11 Bim Knockdown

To inhibit Bim translation using siRNA, a mixture of 4 different siRNA duplexes (Catalog no. M-004383-02, Thermo Scientific Dharmacon, Surrey, UK) targeting all three major transcript variants of *Bim* (*Bim-EL, Bim-L and Bim-S*) was used.

As a control, non-targeting siRNA (Catalog no. D-001210-02, Thermo Scientific Dharmacon) was also used. For each transfection, 1 x 10^7 CLL cells were resuspended in 100 µl transfection solution from a Human B cell Nucleofector Kit (Amaxa AG/Lonza, Cologne, Germany) and mixed with 0.5 nmol of Bim siRNA duplexes or 0.5 nmol of non-specific control siRNA before electroporation on the Nucleofector (Amaxa AG/Lonza) using program X-03. Cells were then mixed with 0.9 ml of pre-warmed medium and cultured overnight at 37^0C. The cells were subsequently incubated at a density of 5 x 10^6 cells/ml, with or without Dex, for a further 48h.

5.3 Results

5.3.1 Bim expression and regulation, and activation of downstream apoptotic effectors

As *Bim* mRNA was shown to be upregulated by Dex in both sensitive and resistant CLL samples (Figure 3.3.6), it was important to identify whether the same was true of Bim protein, given the previously reported importance of Bim induction to GC-induced killing (Bachmann, Gorman et al. 2005, Lu, Quearry et al. 2006, Bouillet, Metcalf et al. 1999, Abrams, Robertson et al. 2004, Erlacher, Michalak et al. 2005, Lopez-Royuela, Balsas et al. 2010). Time points for incubation were chosen so as to allow for a delay between gene transcription and protein accumulation. Given that *Bim* transcript levels had been measured at 6h and 21.5h, it was decided to measure Bim protein levels at 12 and 24h.

As is shown in Fig 5.3.1, Bim protein upregulation was apparent in the majority of CLL samples tested at both 12h and 24h, regardless of sensitivity grouping, though it also accumulated in the absence of Dex treatment, as indicated by the higher levels observed at 12h and 24h compared with 0h in individual samples (Figure 5.3.1 A). Induction of Bim-EL and Bim-S was identifiable in most samples [P=0.002 Bim-EL (12h); P=0.002 Bim-EL (24h); P=0.05 Bim-S (12h), P=0.01 Bim-S (24h)], whilst Bim-L induction was less consistent across the 12 samples at both timepoints (P>0.05).

To test the idea that fold-induction of Bim protein might be higher in the sensitive group, the two groups were compared (Figure 5.3.2). A statistical difference in fold-induction of the three isoforms of Bim between the two sensitivity groups

was not detectable (P>0.05). This may not be surprising given that mere upregulation of Bim is insufficient for apoptosis in certain lymphoma cells (Kfir, Sionov et al. 2007). However, some studies have previously suggested that failure to induce Bim causes GC resistance (Bachmann, Gorman et al. 2005). Despite these observations, at 24h, Bim-EL fold-change did appear to be skewed towards the sensitive group, and so extent of Bim-EL induction may contribute to Dex response in certain sensitive samples (Figure 5.3.1 B). To strengthen this data, separate experiments were performed on 4 samples of each sensitivity group after 48h incubation (Figure 5.3.2 D/E). In agreement with the data from earlier timepoints, Bim-EL was induced in all samples tested at 48h.

Of the three Bim isoforms measured (Figure 5.3.1), it was apparent that Bim-EL was the most consistently upregulated, with a tendency for the sensitive CLL samples to show higher fold-induction of this isoform than the resistant samples (Figure 5.3.1 C). This trend for Bim-EL to be upregulated less prominently in GC-resistant CLL samples may have contributed to GC resistance in these samples. ERK, which is associated with pro-survival signalling (Section 1.6.6), has been reported to phosphorylate and downregulate Bim-EL (Ley, Ewings et al. 2005, Fukazawa, Noguchi et al. 2004). Therefore, the impaired GC-induced Bim upregulation seen in some GC-resistant CLL samples might have resulted from higher constitutive ERK activation, or higher ERK activity post-Dex in these samples. To test this idea, ERK1/2 activation in resistant and sensitive samples was compared by comparison of the expression of the activated phosphorylated version of the kinase with total expression.

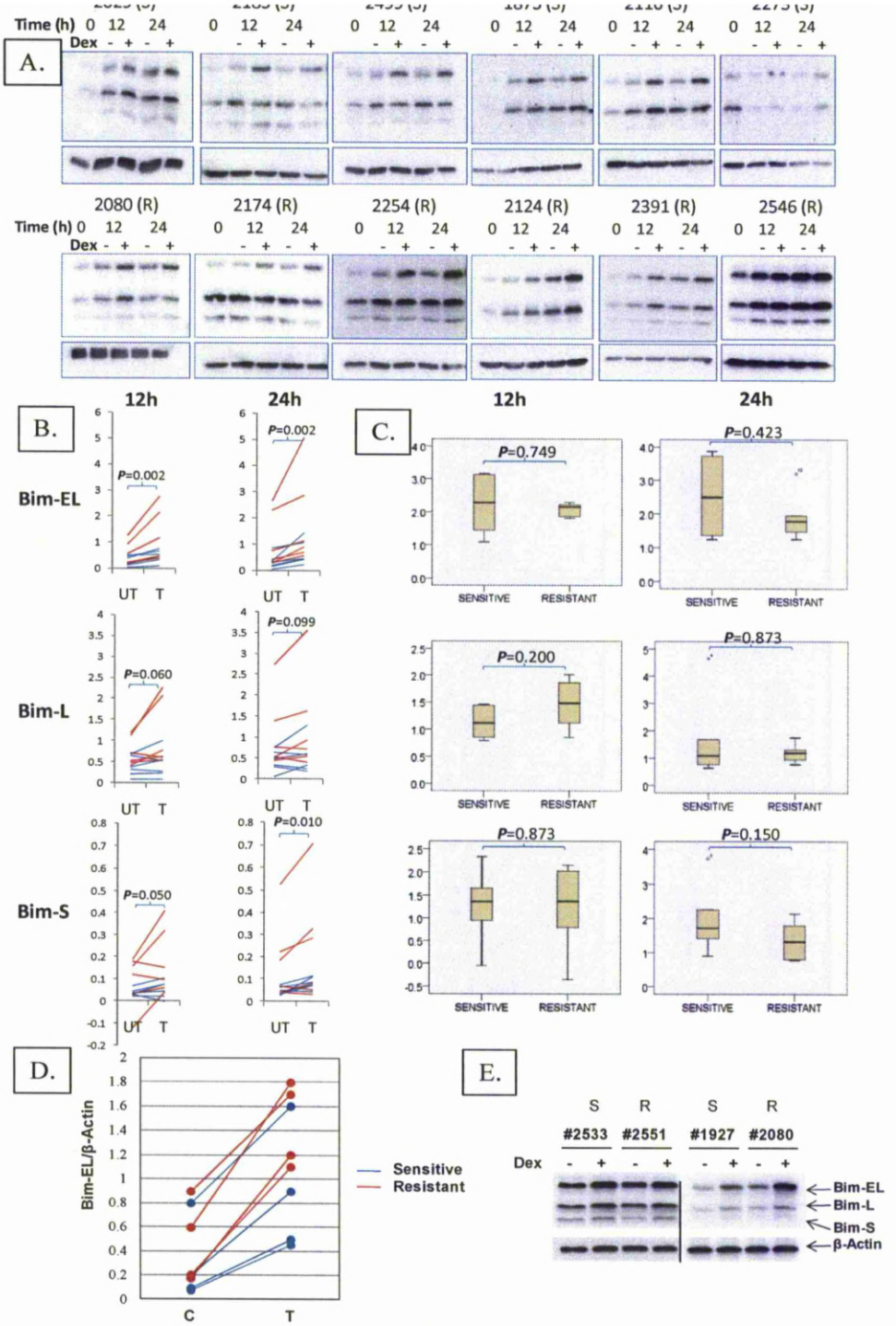


Figure 5.3.1. Bim protein expression is increased by Dex in both sensitive and resistant samples. A. 6 sensitive and 6 resistant samples were cultured w/wo 100nM Dex for 0, 12, and 24h. The 3 protein isoforms (EL, L and S) can be seen on the Western blots at 23, 15, and 12kDa, respectively. Actin is positioned at 42kDa. B. Densitometry for each Bim isoform relative to Actin at 12 and 24h. Blue lines represent sensitive samples, whilst red lines represent resistant samples. ***P***-values of Wilcoxon tests for change are as follows: Bim-EL ***P***=0.002 (12h), ***P***=0.002 (24h); Bim-L ***P***=0.060 (12h), ***P***=0.099 (24h); Bim-S ***P***=0.050 (12h), ***P***=0.010 (24h). C. Fold change for each isoform upon Dex treatment. ***P***-values for Mann-Whitney *U* test for group difference are as follows: Bim-EL ***P***=0.749 (12h), ***P***=0.423 (24h); Bim-L ***P***=0.200 (12h), ***P***=0.873 (24h); Bim-S ***P***=0.873 (12h), ***P***=0.150 (24h). D. Densitometry for independent experiments to demonstrate Bim-EL protein induction at 48h in both sensitive and resistant samples. E. Representative Western blot from those used in "D".

As Figure 5.3.2 B demonstrates, sensitive samples show consistent downregulation of p-ERK at both 12h and 24h ($P<0.05$), whilst p-ERK appears to be stable or be induced slightly by Dex in resistant samples at 12h, and is downregulated in 4/6 by 24h. In the absence of Dex treatment there seems to be a non-significant trend for ERK activation to be higher in sensitive samples, whereas in the presence of Dex, ERK activation is higher in resistant samples (Figure 5.3.2 C). Combining the two measurements to yield fold-change results in a significant difference in ERK activation between the two groups ($P=0.028$, 12h; $P=0.028$, 24h), with median fold change in ERK activation following Dex treatment more than two-fold higher in the resistant group at both timepoints. However, the difference in fold-change between the two groups appears to be mostly attributable to higher baseline ERK activation in sensitive samples. Nonetheless, following Dex treatment at 24h, 5/6 sensitive samples display p-ERK/ERK ratios below that of all 6 resistant samples (Figure 5.3.2 B), which suggests that higher ERK kinase activity post-Dex treatment may contribute to the tendency for resistant CLL samples to display less Bim-EL induction at 24h (Figure 5.3.1 C).

Since MEK is the kinase responsible for activating ERK by phosphorylation, its inhibition leads to ERK deactivation. Furthermore, MEK inhibitors have been shown to potentiate Dex lethality in ALL cells via Bim (Rambal, Panaguiton et al. 2009). Therefore, in order to test the involvement of ERK in GC resistance further, the effect of a MEK inhibitor on GC-induced Bim upregulation and cytotoxicity was investigated (Figure 5.3.3).

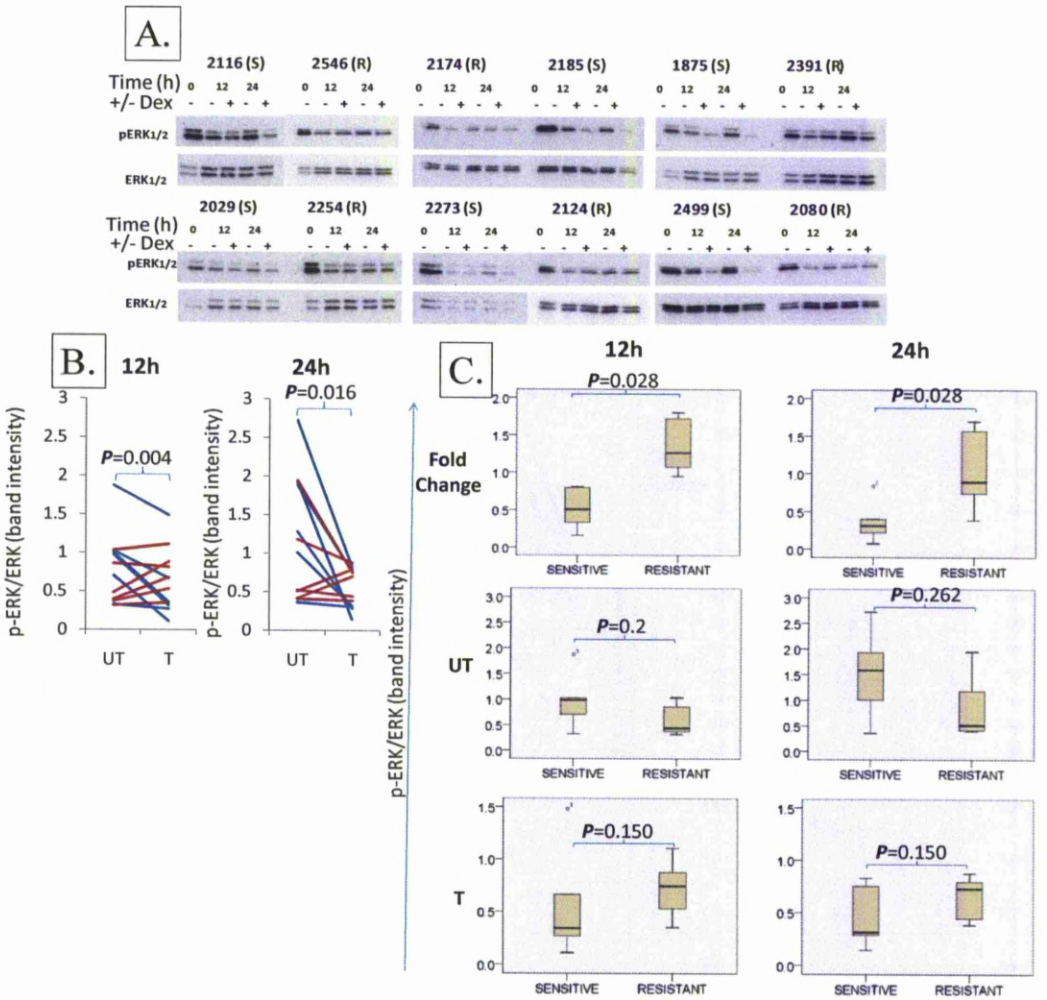


Figure 5.3.2. p-ERK/ERK ratio is reduced by Dex treatment in sensitive samples more so than in those that are resistant. A. Western blots for 6 samples from each sensitivity group. ERK1 can be seen at 44kDa, whilst ERK2 at 42kDa. B. Densitometry for p-ERK1/2 normalised to ERK1/2. Blue lines represent sensitive samples whilst red lines represent resistant. ***P***-values represent Wilcoxon test for only the sensitive samples: ***P***=0.004 (12h), ***P***=0.016 (24h). C. Grouped data comparisons. ***P***-values represent Mann-Whitney *U* test: Fold-change ***P***=0.028 (12h), ***P***=0.028 (24h); Untreated ***P***=0.2 (12h). ***P***=0.262 (24h); Dex-treated ***P***=0.150 (12h), ***P***=0.150 (24h).

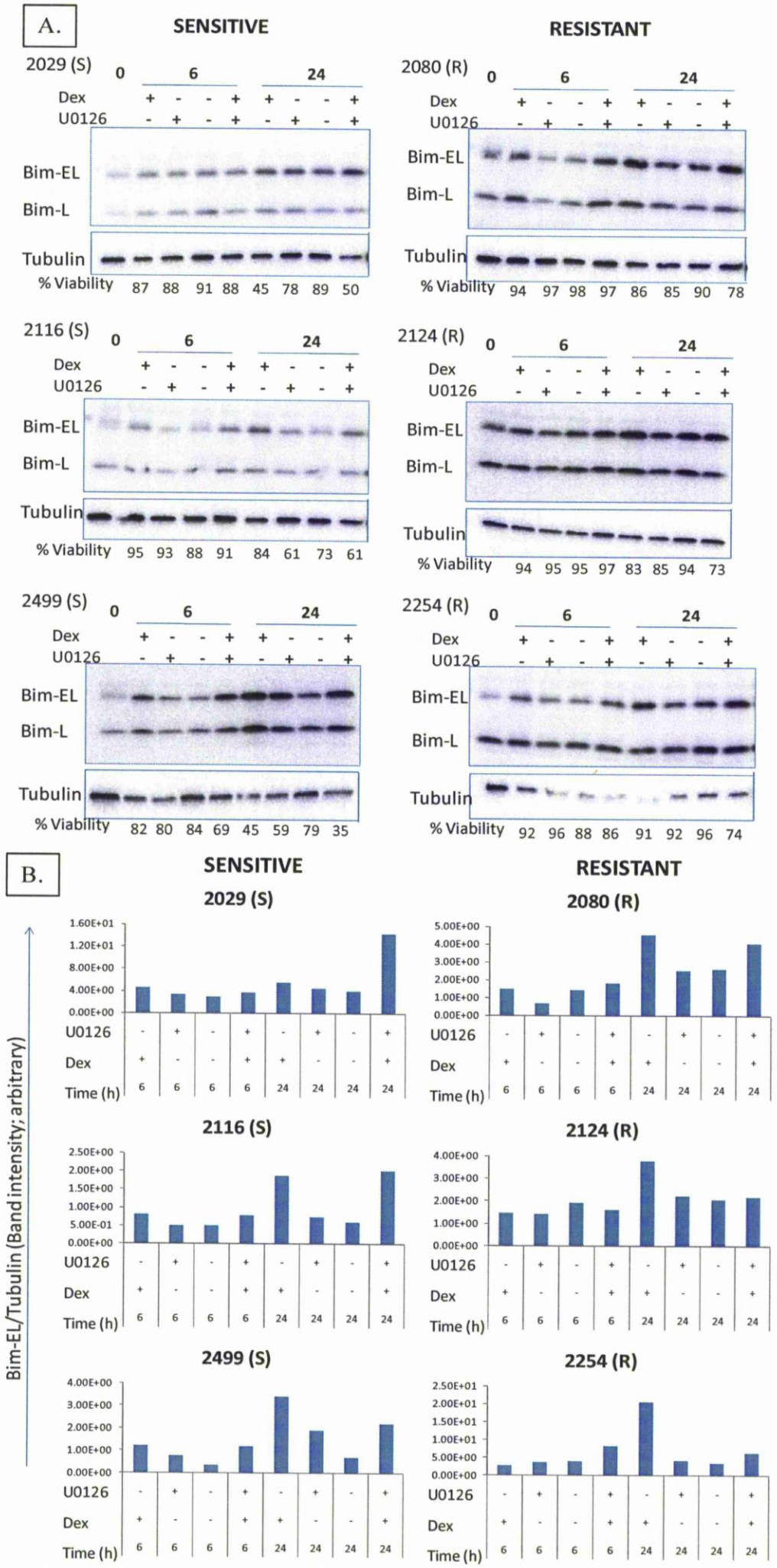
Three samples from each group were cultured with MEK inhibitor and/or Dex to identify whether MEK inhibitor could clearly facilitate Dex-induced killing of resistant samples, and to identify potential cross-resistance. As expected, both sensitive and resistant samples induce Bim-EL in response to Dex (Figure 5.3.3 B), and this effect was much clearer at 24h than 6h, though induction of Bim-EL in sample 2029 was relatively weak. However, a consistent induction of Bim-EL by MEK inhibitor was not detected, and only 2499—a sensitive sample—appeared to demonstrate this phenomenon. MEK inhibitor did not appear to sensitise GC-resistant CLL samples to GC-induced killing (5.3.3 C). This argues against the hypothesis that GC resistance in this study resulted from downregulation of Bim-EL by high ERK activity. Furthermore, MEK inhibitor was selectively cytotoxic to GC-sensitive CLL cells, possibly suggesting that sensitivity to GCs and MEK inhibition are mediated by shared mechanisms.

Bim protein has been shown to be induced in both sensitive and resistant samples in response to Dex (Figure 5.3.1). Therefore, modification of Bim, its sub-cellular localisation, or coassociation with Bim supressors could contribute to a failure of Bim to induce apoptosis in resistant samples (Puthalakath, Strasser 2002). However, prior to studying GC-resistant CLL samples to identify such regulation, it was first important to show that downstream effectors of apoptosis were activated by Dex treatment to a greater extent in sensitive than resistant samples. This would rule out downstream defects in death signalling as potential resistance mechanisms.

As described in Section 1.7.7, Bax and Bak are cytosolic proteins that undergo conformational changes and oligomerisation as a critical activating step of apoptosis in CLL (Dewson, Snowden et al. 2003, Vogler, Dinsdale et al. 2008). Moreover, a strong correlation has previously been observed between the number of Dex-induced apoptotic CLL cells and the percentage of cells stained with antibodies that recognise active Bax and Bak conformation (Bellosillo, Villamor et al. 2002). Therefore, it would be expected for Bax and Bak to be mediators of GC-induced apoptosis in this study.

First of all, a screen of basal t0 expression of Bax and Bak proteins was performed (Figure 5.3.4). In theory, higher baseline expression of Bax and Bak would provide more molecular targets for death inducing Bcl-2 family proteins such as Bim, thus making samples more prone to apoptotic stimuli.

As shown in Figure 5.3.4, the abundance of Bax and Bak protein did not differ between sensitive and resistant samples ($P>0.5$). This observation is consistent with a previous study of 39 CLL samples that showed no relationship between reduced Bax levels and *ex vivo* resistance to corticosteroids ($P>0.5$), despite a correlation existing between Bax levels and traditional therapies; anthracyclines, alkylating agents and vincristine (all $P<0.5$) (Bosanquet, Sturm et al. 2002).


A.
SENSITIVE
RESISTANT
2029 (S)
2080 (R)
2116 (S)
2124 (R)
2499 (S)
2254 (R)
0
6
24
Dex
U0126
Bim-EL
Bim-L
Tubulin
% Viability
87 88 91 88 45 78 89 50
94 97 98 97 86 85 90 78
95 93 88 91 84 61 73 61
94 95 95 97 83 85 94 73
82 80 84 69 45 59 79 35
92 96 88 86 91 92 96 74
B.
SENSITIVE
RESISTANT
2029 (S)
2080 (R)
2116 (S)
2124 (R)
2499 (S)
2254 (R)
Bim-EL/Tubulin (Band intensity; arbitrary)
U0126
Dex
Time (h)

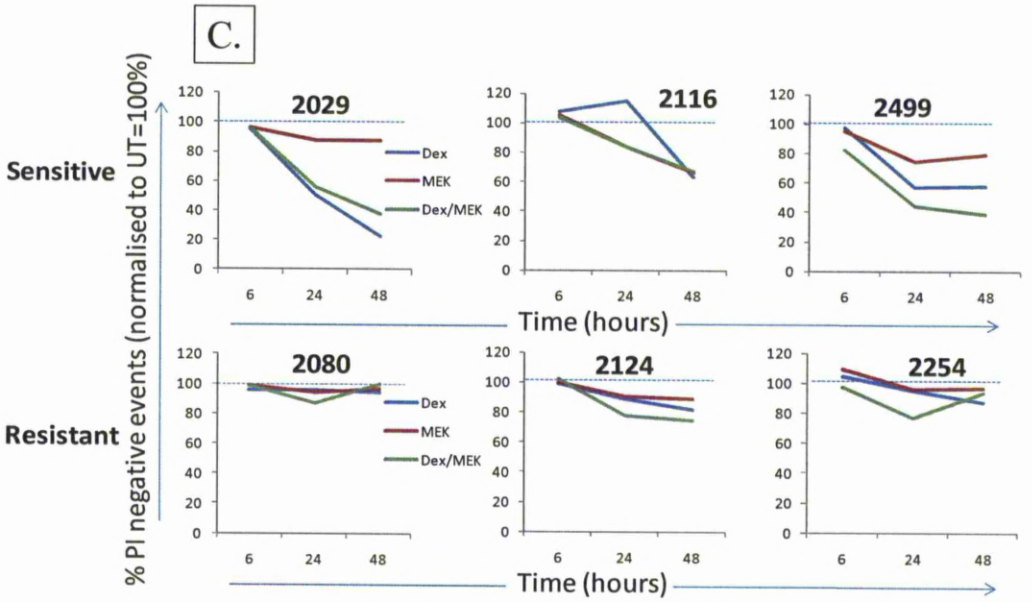


Figure 5.3.3. MEK inhibitor does not sensitise GC-resistant samples to Dex but does kill 2/6 samples effectively, both of which are Dex sensitive. A. Western blots showing Bim-EL, Bim-L and tubulin (normaliser) expression, and viability data. B. Densitometry for "A". C. Viability over 48h. Viability represents PI exclusion.

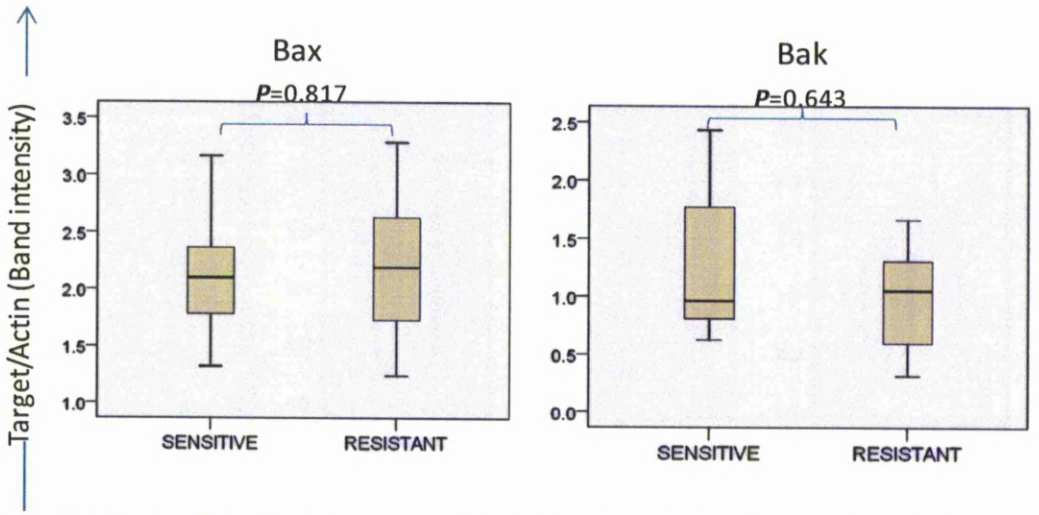


Figure 5.3.4. Basal (t0) expression of Bax and Bak by Western blot (7 sensitive vs. 8 resistant). Densitometry was normalised to that of Actin expression. There was no significant difference in baseline expression of these two proteins between sensitivity groups. ***P***=0.817 and 0.643, respectively, by Mann-Whitney *U* test.

Having established that the quantity of Bax and Bak protein was similar in sensitive and resistant samples before treatment, levels of activated Bax and Bak were then measured in representative samples incubated with and without Dex. Levels of the active isoforms relative to total protein were quantified in order to gain insight into activity. Bax and Bak activation was measured by immunoprecipitation using an antibody that detected an epitope only present on the active form of Bax (Bax IgG_1 6A7, Sigma) or Bak (IgG_2A Ab-1). Western blots were then performed for the immunoprecipitated protein and probed using standard polyclonal Bax or Bak antibody.

For clarity, immunoglobulin heavy and light chains are labelled, and 5% input and output of the immunoprecipitation are shown (Figure 5.3.5 A-C). Bax was detected in the post-IP as well as the pre-IP fraction as the immunoprecipitation only removes active Bax from a sample, and because no IP is 100% efficient. Both a substantial increase in active Bax levels and cell death was observed when sensitive samples were treated with Dex, whilst the effect was much less marked when resistant samples were treated with Dex. Therefore, it can be concluded that Bax activation does not occur in resistant samples to the same extent that it occurs in sensitive samples. A similar trend was observed when Bak activation was measured by IP in a sensitive and resistant sample (Figure 5.3.5 D). Hence, the failure of Bim to induce killing is the result of events upstream of Bax and Bak activation.

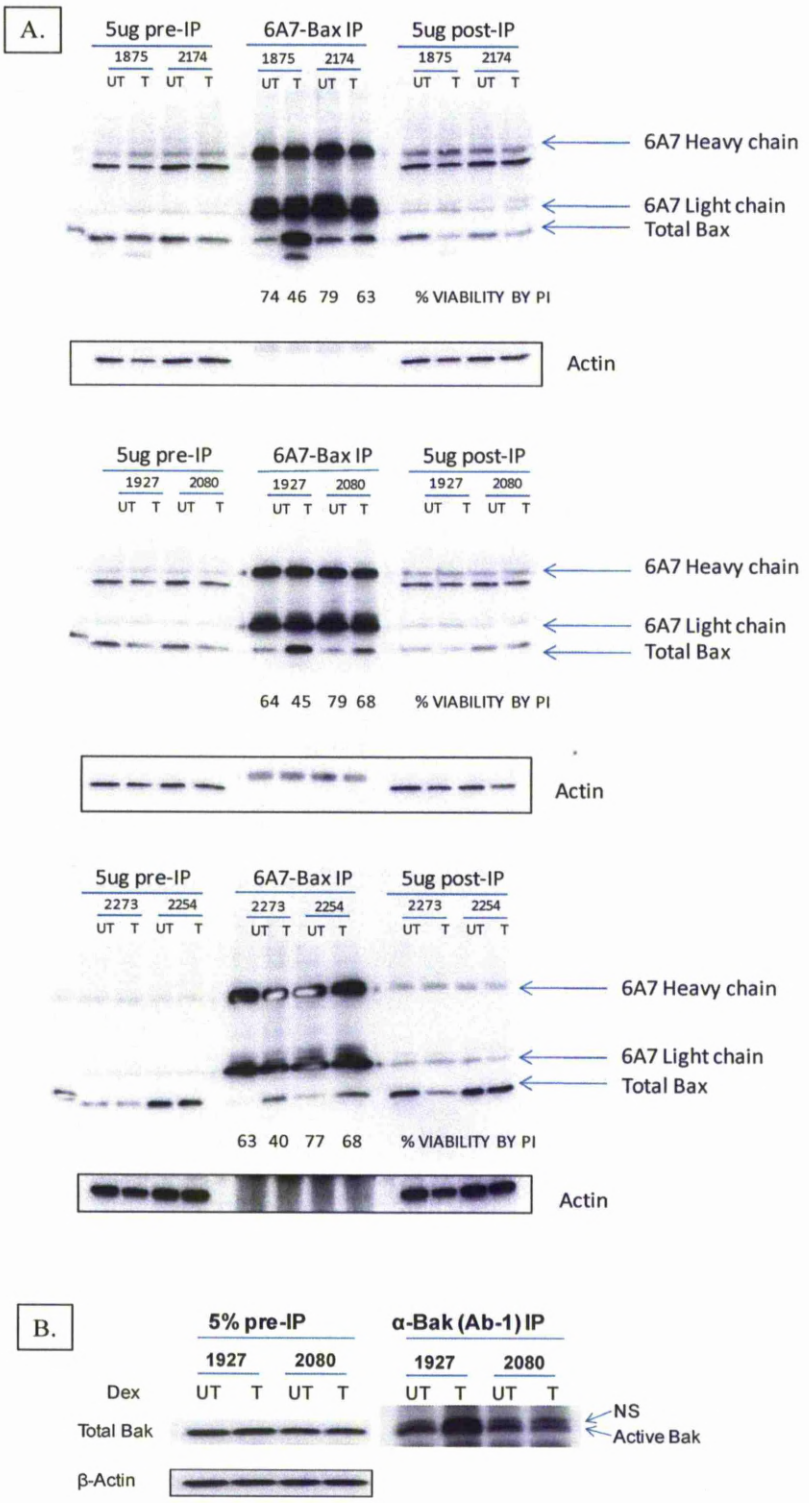


Figure 5.3.5 Bax and Bak activation is skewed towards GC-sensitive samples following Dex treatment. Active Bax was immunoprecipitated and then loaded onto Western blots alongside 5% IP input and 5% output. Blots were stained with an antibody that detects total Bax. A. 3 blots, each of which features 1 sensitive (left) and 1 resistant (right) sample. Viability is measured by PI exclusion. B. Bak was also determined to be activated in response to Dex in a sensitive but not a resistant sample. NS = non-specific band.

5.3.2 Expression and binding of Bim antagonists

Figure 5.3.1 A not only showed Bim upregulation in sensitive and resistant samples; but it also suggested a difference in baseline expression of Bim protein; i.e., Bim appeared to be higher at baseline in resistant than sensitive samples. Despite a difference in ERK activation possibly explaining this observation in terms of Bim-EL expression, the other isoforms could not be ignored. If a difference in expression of all three isoforms was shown to exist between the two sensitivity groups then it could be speculated that the post-translational control of Bim in resistant samples was skewed towards survival. To clarify this, t0 samples from the previously screened 6 sensitive and 6 resistant samples were immunoblotted on a single gel (Figure 5.3.6 A).

As suspected, there was a higher basal t0 expression of Bim in resistant samples than sensitive, with median expression in resistant samples more than double that of sensitive samples. Group difference in expression was significant ($P<0.05$) for all of the Bim isoforms individually (5.3.6 B) or when grouped (5.3.6 C). Bcl-2 is a known attenuator of Bim in CLL cells (Del Gaizo Moore, Brown et al. 2007) that is overexpressed in CLL, and is a key element of CLL apoptotic resistance (Pepper, Thomas et al. 1999b). Therefore, it was hypothesised that resistant samples might possess higher baseline expression of Bcl-2, which may serve to protect CLL cells from Dex-induced Bim induction by sequestration. A Western blot parallel to Figure 5.3.4 A was performed to indicate whether this might be the case (Figure 5.3.4 D). Though the correlation between Bcl-2 and Bim expression is too weak to suggest that their levels at t0 are directly related (Figure 5.3.4 F), there is a tendency for resistant samples to express higher levels of Bcl-2 than

sensitive samples (Figure 5.3.4 E). This suggests that there is more Bcl-2 available in these samples that might act as a sink for Bim.

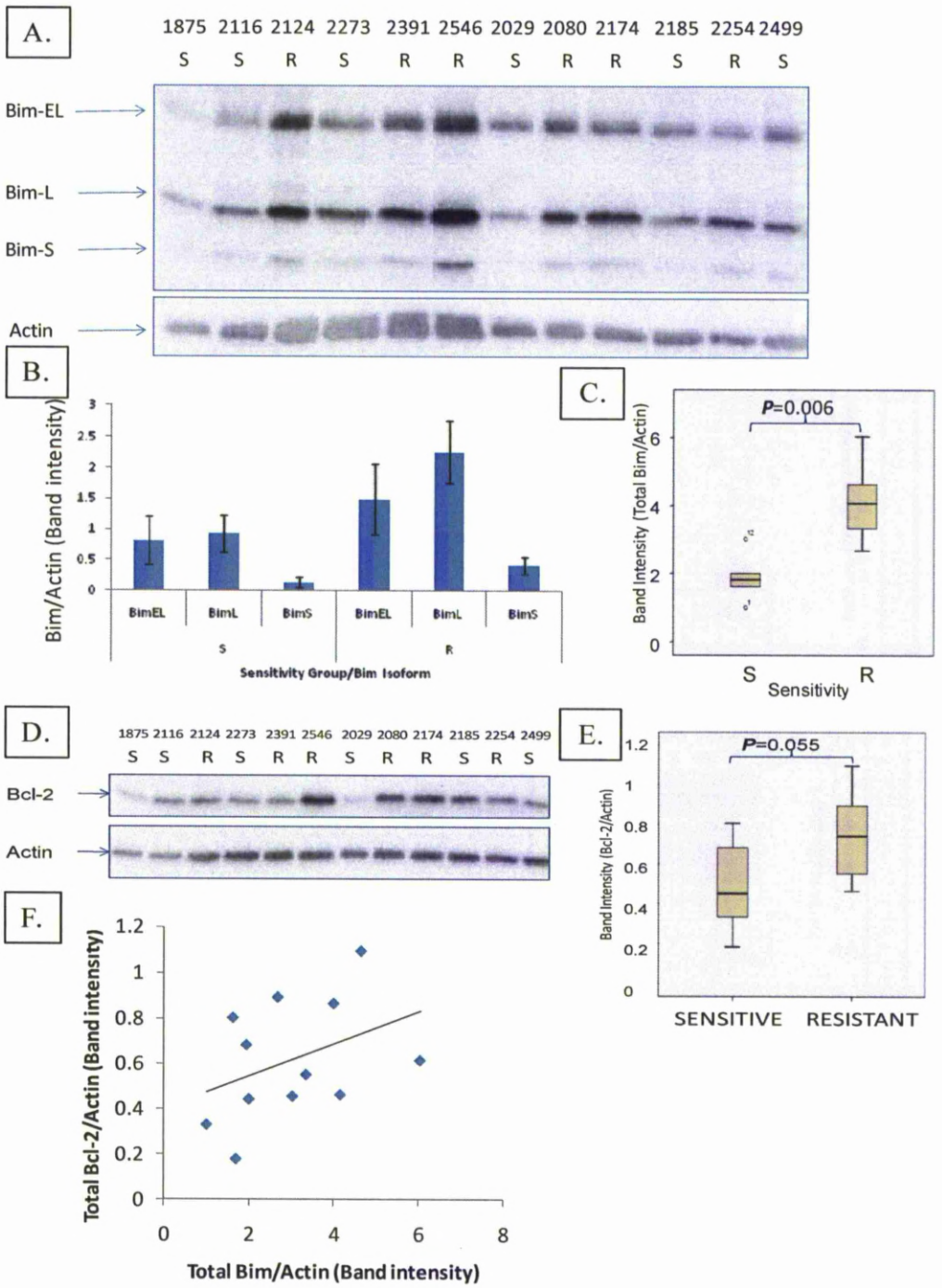


Figure 5.3.6. Bim expression appears to be higher at t0 in GC-resistant than GC-sensitive samples, and Bcl-2 also shows a tendency to be more highly expressed in resistant samples. A. SDS-lysates were separated by electrophoresis and Western blotting was performed to detect Bim-EL (23kDa), Bim-L (15kDa), Bim-S (12kDa) and Actin (42kDa). B. Densitometry analysis shows that all 3 Bim isoforms were more highly expressed in the sensitive group using Mann-Whitney *U* test (B: Bim-EL ***P***=0.037, Bim-L ***P***=0.004, Bim-S ***P***=0.004). C. Total Bim is more highly expressed in resistant samples (***P***= 0.006 by Mann-Whitney *U* test) D. Western blotting shows expression of Bcl-2 (26kDa) and Actin (42kDa). E. Bcl-2 expression in resistant samples is generally higher than that of sensitive samples (***P***=0.05 by Mann Whitney *U* test). F. Bcl-2 and Bim expression appear to loosely correlate (R^2=0.161, ***P***=0.196 by Linear Regression).

To provide further evidence that antiapoptotic proteins were more highly expressed in resistant samples, an early timecourse was performed (8, 12, and 16h) within which Dex treatment was included (Figure 5.3.7). Relatively early timepoints were chosen for this study in order to negate the potentially biasing effects of culturing sensitive and resistant samples for longer. As well as Bcl-2, another critical mediator of CLL cell survival—Mcl-1—was measured, which also has been reported to associate with Bim in B cells (Gomez-Bougie, Bataille et al. 2005). Furthermore, Mcl-1 expression has been associated with failure to achieve complete remission in response to fludarabine and chlorambucil in CLL (Kitada, Andersen et al. 1998). CLL samples were cultured w/wo Dex for 8, 12, and 16h. As Figure 2.3.4 showed that sensitive samples were undergoing apoptosis as early as 4h following Dex treatment, these relatively early timepoints were selected to negate the possibility of death-induced bias.

Consistent with Figure 5.3.6, Bcl-2 is overexpressed in resistant samples relative to sensitive samples, though statistical validity is imperfect over time (Figure 5.3.7 C). Furthermore, Dex does not appear to alter Bcl-2 expression (Figure 5.3.7 B). Therefore, overexpression of Bcl-2 may play a role in preventing GC-induced killing. This opposes previous observations of 39 CLL samples *in vitro*, which showed that Bcl-2 did not associate with drug sensitivity at all (Bosanquet, Sturm et al. 2002). Mcl-1 levels before and after treatment are of a similar range regardless of sensitivity group (Figure 5.3.7 B). Therefore, neither baseline expression level nor failure of downregulation of Mcl-1 expression (Figure 5.3.7 C) appears to block GC action in resistant samples. However, the observed consistent downregulation of Mcl-1 in response to Dex is interesting.

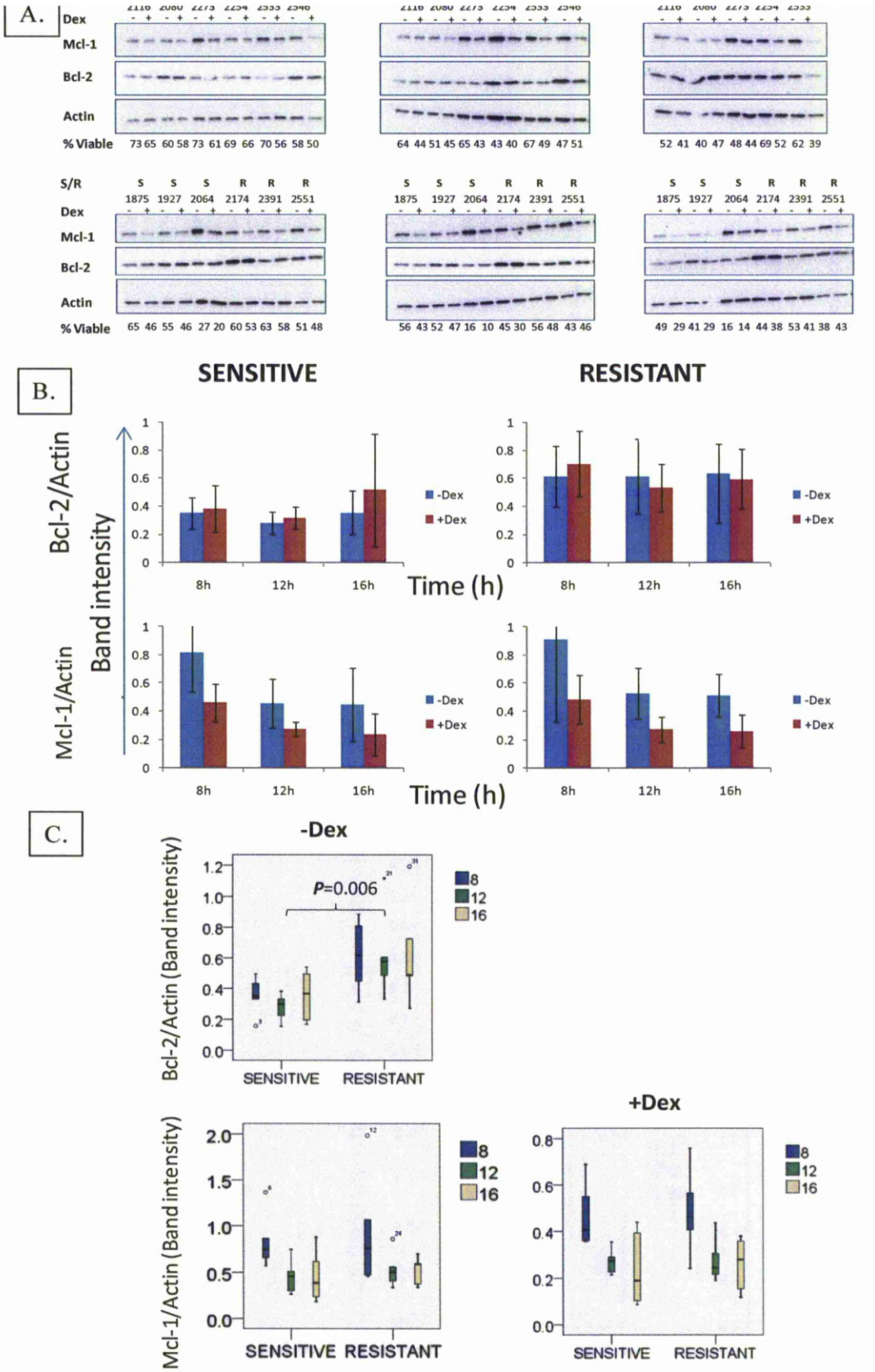


Figure 5.3.7. Bcl-2 expression is higher in GC-resistant than GC-sensitive samples, whilst Mcl-1 levels are similar in both sensitivity groups. A. Western blots for Mcl-1 (rabbit PAb,42kDa), Bcl-2 (mouse MAb, 29kDa), and Actin (40kDa) in 6 sensitive and 6 resistant samples at 8h, 12h, and 16h. Viability was determined by $DiOC_6$/PI. B. Histograms representing mean expression of Bcl-2 and Mcl-1 relative to Actin by densitometry. C. Grouped data stem and leaf plots. Mann-Whitney *U* test ***P***-values for sensitive versus resistant are as

follows: Bcl-2 UT 0.055 (8h), 0.006 (12h), 0.201 (16h); Mcl-1 UT 0.749 (8h), 0.423 (UT), 0.465 (UT); Mcl-1 T: 0.522 (8h), 0.749 (12h), 0.855 (16h).

Mcl-1 expression is rapidly altered by transcription and protein stability by various environmental cues. It has a rapid turnover and can be targeted by ubiquitination for proteasomal degradation. During apoptosis, Mcl-1 is an efficient caspase substrate (Clohessy, Zhuang et al. 2004). However, Figure 5.3.7 B displays ~50% reduction in Mcl-1 as early as 8h post-Dex culture, whilst death induction in the sensitive samples as measured by $DiOC_6$/PI was measured at only 18%. Taken together, these two points suggest it is unlikely that the Dex-induced reduction of Mcl-1 in both groups was simply a result of caspase-mediated cleavage. To explore this issue further, 4 sensitive samples were cultured for 8h in the absence or presence of both Dex and a pan-caspase inhibitor, Z-vad.fmk (Figure 5.3.8).

In all samples, Mcl-1 was downregulated by Dex, but caspase inhibitor did not rescue CLL cells from this effect, despite its effectiveness in preventing PARP cleavage in 4/4 samples (Figure 5.3.8 A/B) and apoptosis in 3/4 samples (Figure 5.3.8 C). Therefore, Mcl-1 downregulation results from a non-caspase dependent mechanism following CLL treatment with Dex, at least in the early phase of GC action. Furthermore, as Mcl-1 downregulation occurs in the absence of killing, it is not likely that Mcl-1 is important to Dex-induced killing of CLL cells, at least at early timepoints.

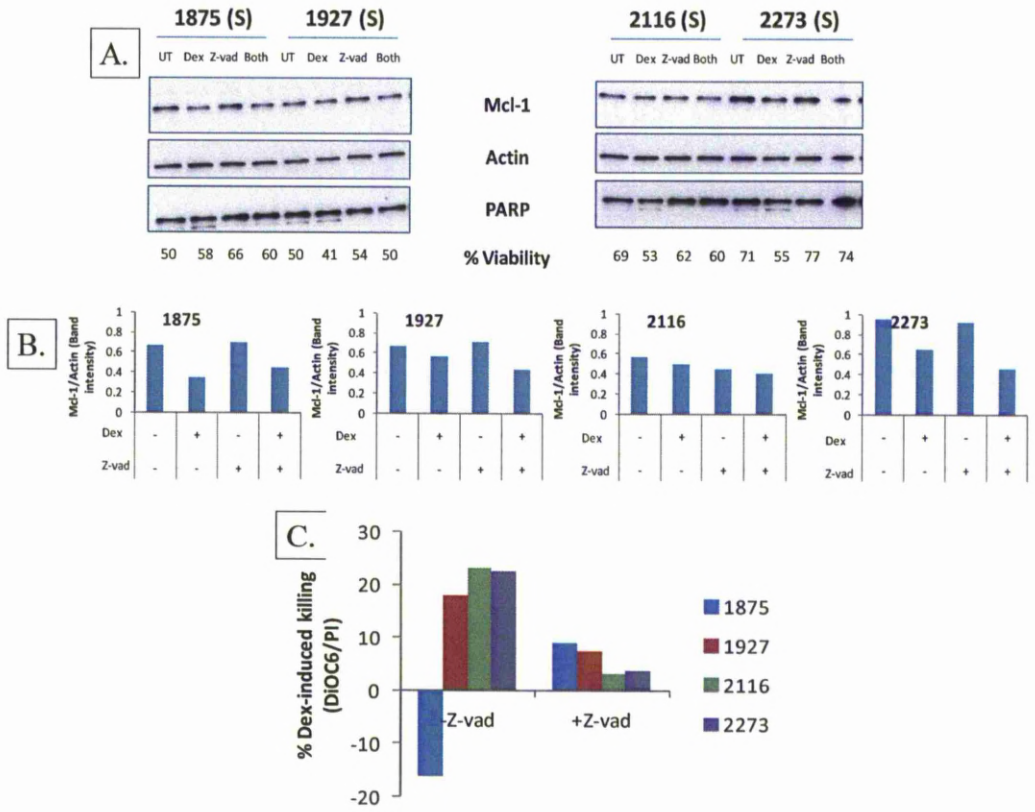


Figure 5.3.8 At 8h Z-vad.fmk confers resistance to Dex upon 3/4 sensitive samples despite evidence of Mcl-1 downregulation. Samples were incubated in the presence or absence of 100nM Dex and z-vad.fmk (1h pre-incubation) for 8h. A. Western blots (10μg per sample) showing Mcl-1 (rabbit PAb) and Actin (loading control) expression, and PARP/cleaved-PARP as a positive control for caspase activation. Viability was measured using $DiOC_6$/PI. B. Densitometry for "A". C. Dex-induced killing data for each sample (***P***=0.61, paired *t* test).

Though Mcl-1 downregulation by Dex could warrant further exploration, the overall purpose of this thesis is to identify signalling events that differ between samples that respond well and those that do not to treatment with Dex. Therefore, this avenue of investigation was not further developed. In contrast, Bim appeared to be expressed at a higher level in resistant than sensitive samples, and this coincided with Bcl-2 expression (Figure 5.3.6/Figure 5.3.7). Because Bax activation is more marked in sensitive samples (Figure 5.3.5) a failure of indirect or direct Bim activation of Bax could be envisaged in resistant samples. However, the importance of Bim in Dex-induced killing had not been assessed, nor had any coassociation with Bim antagonists. Therefore, siRNA knockdown, which had previously been shown to protect GC-sensitive B-ALL cells from GCs (Abrams, Robertson et al. 2004), was performed to reduce Bim expression in sensitive samples (Figure 5.3.9). If a resistant phenotype could be induced, this would demonstrate a critical role for Bim in Dex-induced killing in CLL. Indeed, Bim-EL was induced by Dex, as expected, in Dex-sensitive CLL samples (Figure 5.3.9 A). Control siRNA had no effect on this induction. However, Bim siRNA clearly reduced the expression of Bim. Concomitantly, killing induced by Dex was reduced to 50% when cells were pretreated with Bim siRNA (Figure 5.3.9 B). Therefore, it can be concluded that Bim-EL is a critical molecule in Dex-induced apoptosis in this experimental system.

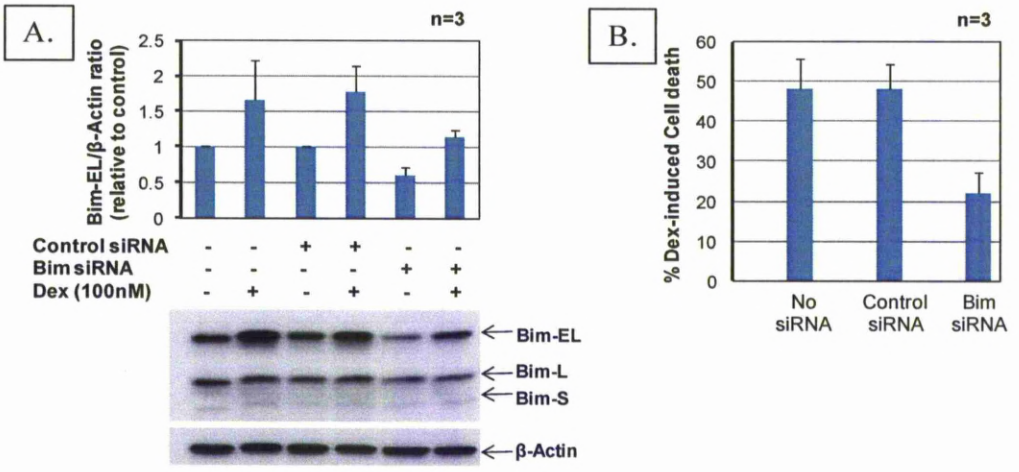


Figure 5.3.9 Knockdown of Bim rescues Dex-sensitive CLL samples from Dex-induced killing. 3 Sensitive samples (1927, 2273, 2422) were pre-treated with Bim siRNA and 24h later were cultured with 100nM Dex for a further 48h. A. A representative Western blot and mean Bim-EL expression for various controls and experimental samples (error bars = standard deviation). B. The effect of control and Bim siRNA on Dex-induced killing (measured by PI exclusion).

Release of Bim-EL and Bim-L from cytoskeletal sequestration can facilitate Bim activation (Puthalakath, Huang et al. 1999, Ley, Balmanno et al. 2003), which has been reported to involve phosphorylation by JNK and p38 (Ley, Ewings et al. 2005)(Chen, Zhou 2004, Cai, Chang et al. 2006). More recently, GSK3 has been reported to activate Bim through a direct interaction (Spokoini, Kfir-Erenfeld et al. 2010). Contrastingly, Bim-EL is predominantly serine phosphorylated in murine lymphoma lymphocytes and thymocytes, which has been reported to prevent, rather than induce, Bim-induced apoptosis (Seward, von Haller et al. 2003). Thus, Bim activation may be regulated both positively and negatively by phosphorylation. Due to a lack of clarity in the literature regarding the nature of Bim phosphorylation and molecular activation, it was decided to focus on Bim binding partners as potentially hindering Dex-upregulated Bim.

Bim has been shown to bind a number of different proteins in B-cells (Gomez-Bougie, Bataille et al. 2005). In normal tonsil B cells Bim was shown to associate with Bcl-2, Mcl-1, and Bcl-xl. The Mcl-1/Bim complex was the most abundant of the three dominant complexes. Additionally, a weak association between Bim-EL and Bim-L with DLC8 was found. As Bim is shown to be upregulated in both sensitive and resistant samples consistently within this investigation it is plausible that one or a combination of antiapoptotic proteins is able to block the proapoptotic function of Bim in resistant samples by physical antagonism and/or sub-cellular sequestration. To test this hypothesis Bim was immunoprecipitated from 3 sensitive and 3 resistant samples at 48h to identify whether the protein coassociated with previously reported antagonists in CLL cells. If association

were detected, the effect of Dex treatment could be determined, and sensitive and resistant samples could be compared.

Before attempting to detect Bim-binding partners in immunoprecipitated samples it was necessary to ensure their presence in the CLL lysates to be used in immunoprecipitation. Bcl-xl was barely detectable in RIPA-soluble and -insoluble fractions by Western blotting relative to CD154-stimulated cells (5.3.10 A), as expected. Contrastingly, DLC8 expression was difficult to detect in RIPA lysates but less so in SDS-lysed RIPA-insoluble pellets (5.3.10 B). This highlights a flaw in using the gentler lysis buffers required for applications such as immunoprecipitation. Moreover, as Bim was clearly detected in the RIPA-insoluble pellet (Figure 5.3.10 A), this fraction of the total Bim was not included in the binding partner assay. Nevertheless, the insoluble pellets consistently contained less protein than the RIPA lysates. DLC8, as well as Bcl-xl were thus excluded from the Bim IP.

Following Bim immunoprecipitation, Mcl-1 was not detectable in the IP fraction, but was apparent in the pre-IP and post-IP fractions (Figure 5.3.10 C), indicating a lack of association between Bim and Mcl-1 in CLL cells. Contrastingly, Bcl-2 clearly co-associated with Bim, and this binding was specific as confirmed by a lack of association with the IP isotype control. Bax was not associated with Bim as indicated by the lack of detection in the IP fraction relative to the lysate and IP supernatant. Together these data support an indirect model of Bax/Bak activation by Bim, and highlight Bcl-2 as a critical binding partner of Bim in CLL cells.

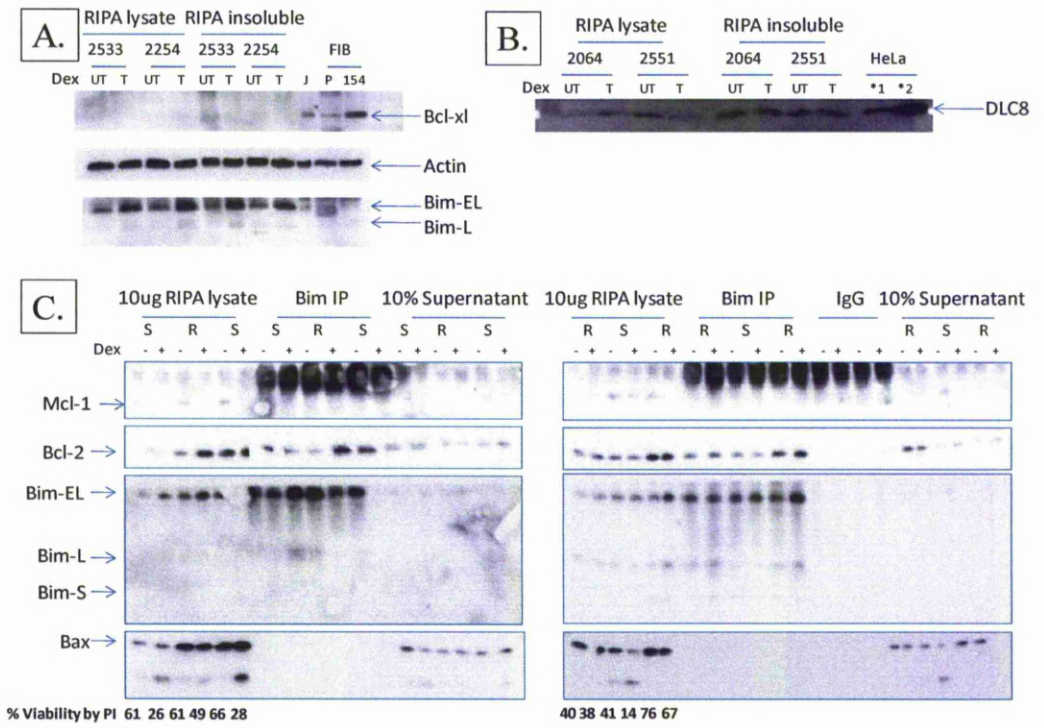


Figure 5.3.10 Screen for Bim-binding partners. 3 sensitive and 3 resistant samples were cultured w/wo Dex for 48h. A. Bcl-xl (26kDa) is neither readily detected by immunoblot in CLL or non-CD154 expressing fibroblasts (P), whilst CLL cells co-cultured with CD-154 expressing fibroblasts (154) do express readily detectable Bcl-xl. Bim is detected in RIPA lysate as well as SDS-lysed RIPA-insoluble pellet (2533=sensitive, 2254=resistant). B. DLC8 (10kDa) is detectable in CLL cells, though RIPA lysis buffer yields only a fraction of the DLC8 that is detected in the RIPA-insoluble pellet (2064=sensitive, 2551=resistant). C. Bim IP of 3 sensitive and 3 resistant samples shows that Bcl-2 (25kDa) appears to co-associate with Bim, whilst Mcl-1 (40kDa) and Bax (21kDa) do not.

Further immunoprecipitation experiments confirmed a lack of association between Bim and Bax, and also Bak (Figure 5.3.11 A/B/C). Once again, Bim-bound Mcl-1 was difficult to detect, whilst Bcl-2 was clearly associated with Bim (Figure 5.3.11 B). A reciprocal IP using Bcl-2 antibody further confirmed the Bim:Bcl-2 interaction (Figure 5.3.11 E).

As the study of the potential Bim antagonists had uncovered only Bcl-2 as a candidate for Bim suppression, and given the apparent overexpression of Bcl-2 in resistant samples, it was clear that resensitisation of resistant samples to Dex should be attempted via inhibition of Bcl-2. ABT-737, a BH3-only mimetic, is a potent killer of CLL cells, and can displace proapoptotic proteins from Bcl-2, Bcl-xl, and Bcl-w (Certo, Del Gaizo Moore et al. 2006), but not from Mcl-1 or Bcl-2A1 (Oltersdorf, Elmore et al. 2005, van Delft, Wei et al. 2006). As this compound has been previously shown to synergise with Dex in CLL cells (Mason, Khaw et al. 2009), it was decided to test for synergy in Dex resistant samples (Figure 5.3.12).

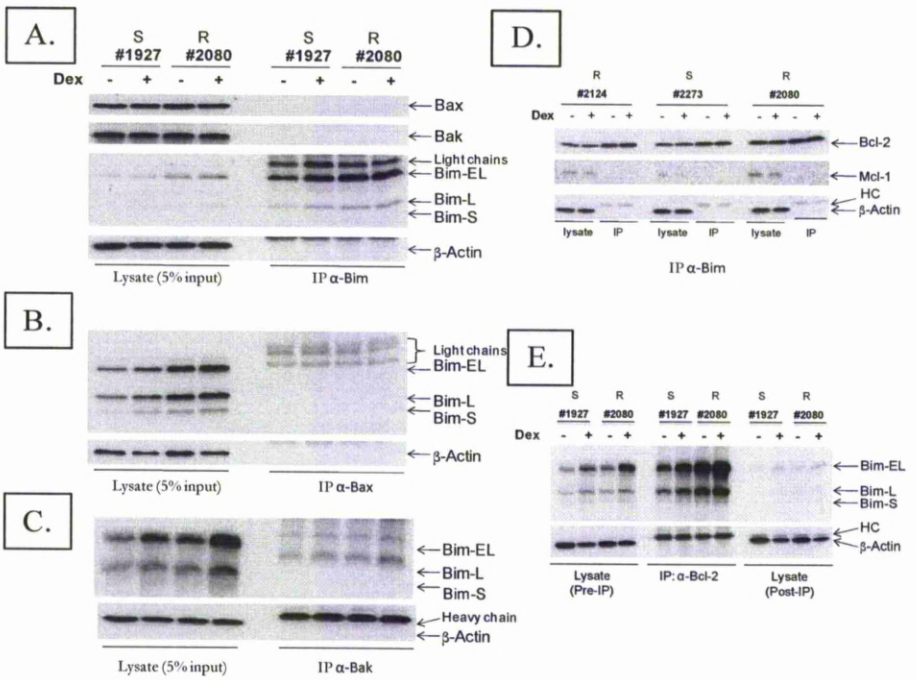


Figure 5.3.11. Bim does not interact directly with Bax or Bak, but is sequestered by Bcl-2 in CLL samples. A/B/C. Western blots to identify coassociation between Bim and Bax/Bak following immunoprecipitation of CLL samples cultured w/wo 100nM Dex for 48h. D/E. Western blots to identify coassociation between Bim and Bcl-2/Mcl-1 following immunoprecipitation of CLL samples cultured w/wo 100nM Dex for 48h.

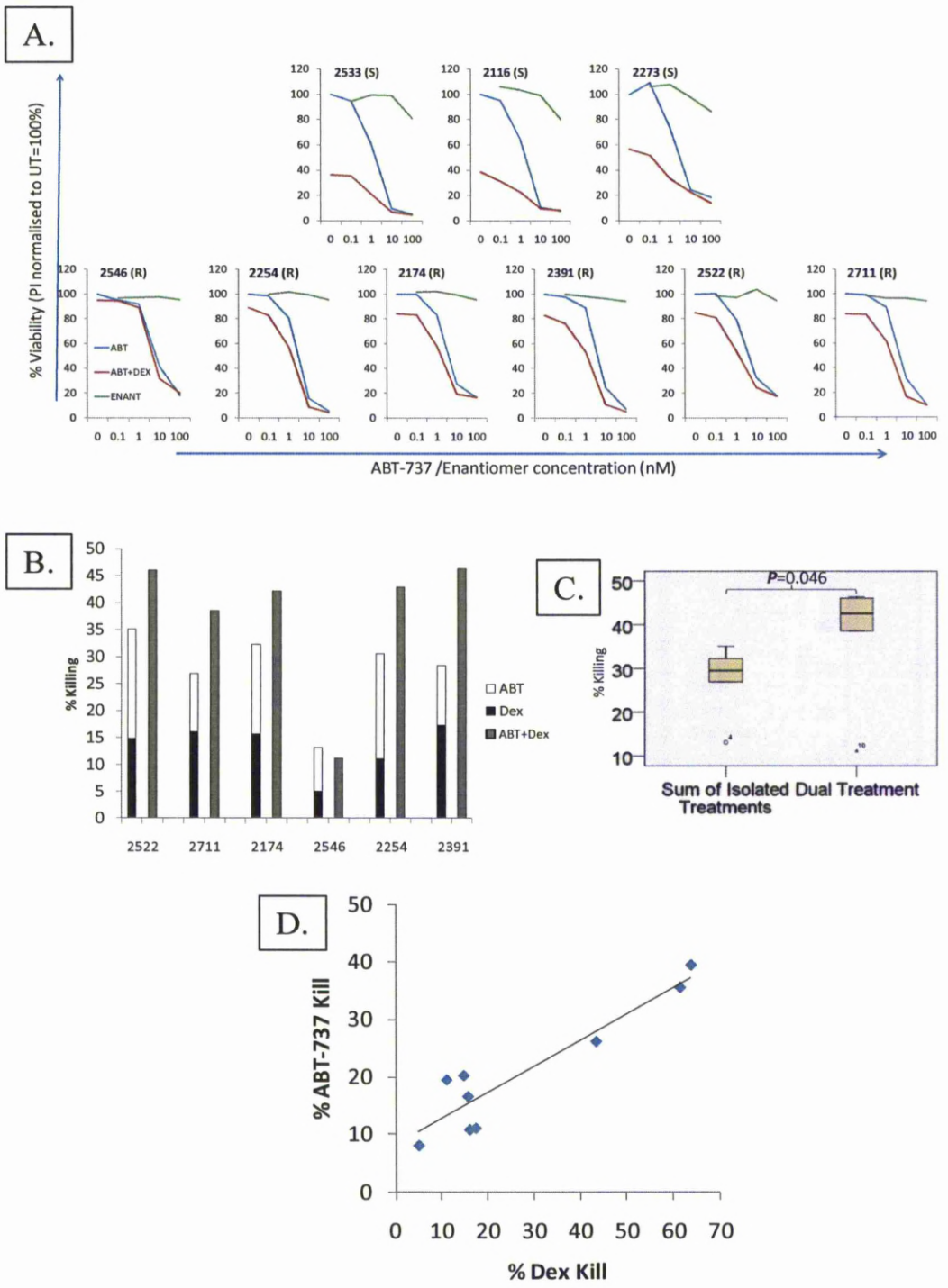


Figure 5.3.12 ABT-737 increases Dex-induced killing beyond that induced by the isolated treatments alone. A. Killing of 6 resistant and 3 sensitive samples by ABT-737 and enantiomer w/wo Dex. B. A comparison of combined killing and mutual killing by Dex and 1nM ABT-737. C. A statistical comparison of the data from "B" (***P***=Mann-Whitney *U* test). D. Dex killing versus 1nM ABT0737 killing (R^2=0.865, ***P***=0.000278).

Figure 5.3.12 suggests that ABT-737 enhances the killing of CLL cells by Dex. 6 resistant and 3 sensitive samples were treated with a range of concentrations of ABT-737 or enantiomer, and were cultured for 48h in the absence or presence of 100nM Dex (Figure 5.3.12 A). All CLL samples were killed effectively by 100nM ABT-737, but sensitivity to this agent correlated with sensitivity to 100nM Dex (Figure 5.3.12 D), possibly implying a shared mechanism of killing. At 1nM ABT-737, resistant samples display some reduction in viability, but still show relative viabilities that are consistently over 70% (Figure 5.3.12 A). Therefore, it was decided to use this concentration of ABT-737 to contrast additive and combined killing of resistant samples by the two agents (Figure 5.3.12 B). The median increase in killing was over 10% when the two agents were combined relative to that achieved by the summation of killing by the two agents in isolation (P=0.046) (Figure 5.3.12 C). Therefore, ABT-737 may have been acting in synergy with Dex. This highlights an important role for Bcl-2 in sequestering Bim as a mechanism of GC resistance in CLL. This is perhaps not a surprising observation given that Bcl-2 sequestration of Bim has been strongly suggested to have importance for CLL cell survival (Del Gaizo Moore, Brown et al. 2007). Moreover, a further previous study has shown that co-incubation of CLL samples with Dex and ABT-737 sensitises CLL samples to the BH-3 only mimetic (Mason, Khaw et al. 2009). However, in conflict with Figure 5.3.12 D, this effect could not be predicted by responses to either agent alone. Notwithstanding these observations, the identification of a role for ABT-737 in sensitising resistant CLL samples to treatment with Dex is novel.

5.4 Discussion

The aim of this chapter was to explore the regulation of downstream effectors of GC-induced killing in CLL. This centred upon the regulation of Bim due to its well-reported role in multiple models of GC-induced killing (Bachmann, Gorman et al. 2005, Lu, Quearry et al. 2006, Bouillet, Metcalf et al. 1999, Abrams, Robertson et al. 2004, Erlacher, Michalak et al. 2005, Lopez-Royuela, Balsas et al. 2010). Bim protein induction was detected in both sensitive and resistant samples, thus GC resistance in this study was not caused by a failure to induce Bim protein. However, subtle differences in Bim expression between sensitive and resistant samples led to an exploration of upstream ERK signalling by virtue of the reported relationship between the two proteins (Ewings, Wiggins et al. 2007). This data did not support a major role for ERK or regulation of Bim-EL expression as a cause of GC resistance. In contrast with some previous studies of T cells and lymphoma cell lines (Rathmell, Lindsten et al. 2002, Salomons, Brady et al. 1997), a difference in the baseline expression of Bax and Bak was not found, though in this investigation the activation of these proteins was clearly related to the induction of killing by GCs. However, it should be noted that the demonstration of Bax and Bak activation in resistant samples by non-GC agents would provide this observation with further substance. The observation of higher expression of Bcl-2 (though only approximately double, with high standard deviation) but not Mcl-1 in resistant samples led to the hypothesis that Bcl-2 sequestration of Bim might be responsible for the failure of Dex-induced Bim to kill resistant samples. Whilst Bcl-2 overexpression has been linked with apoptosis resistance and drug resistance in CLL (Pepper, Thomas et al. 1999b, Pepper, Thomas et al. 1999a), it has not been previously associated with GC

resistance. The importance of Bim was confirmed by the cytoprotective effect of siRNA knockdown in GC-sensitive samples. Although knockout mice of Bim had demonstrated a role for Bim in the GC-induced killing of lymphocytes (Bouillet, Metcalf et al. 1999), this is the first demonstration by gene silencing (siRNA) of the involvement of Bim in CLL GC-induced death. The most interesting aspect of this chapter is the analysis of the role played by Bcl-2 in buffering Bim and making CLL cells with high Bcl-2 levels resistant to Dex-induced apoptosis. A clear association of Bim with Bcl-2, but not other potential Bim-binding partners, was demonstrated. To provide further confidence in the negative results of this particular study reciprocal IPs should be performed. ABT-737 has been previously been shown to facilitate the killing of CLL cells by GCs (Mason, Khaw et al. 2009). In this investigation, co-treatment with Dex and the Bcl-2 inhibitor actually appeared to sensitise resistant samples to GC-induced killing, though formal tests for synergy are yet to be performed. It can therefore be concluded that sequestration of Bim by Bcl-2 is a mechanism that contributes significantly to GC resistance in CLL.

Further experiments would certainly solidify the main conclusions of this chapter. For instance, the effect of overexpression of Bcl-2 in sensitive samples would be highly informative, as would Bcl-2 knockdown. Furthermore, the effect of Bim overexpression on sensitivity could further emphasise the importance of this mechanism in GC resistance in CLL. Despite the observation that Bim predominantly appeared to be bound to Bcl-2 in CLL cells, this observation applied to both sensitive and resistant samples. Furthermore the Bim knockdown negated killing at 48h in sensitive samples, but it remains to be seen whether this

merely corresponded to a delay in death kinetics rather than *bona fide* desensitisation. Thus, it would be informative to follow sample viability beyond 48h post-Dex in sensitive samples that had been pretreated with Bim siRNA. However, this may prove difficult as viability is considerably reduced by the electroporation procedure alone at 72h. Further, whilst Bim silencing may densensitise sensitive samples, this observation does not explain why Bim upregulation in resistant samples was not sufficient to induce cell death. As yet, direct evidence is not available that proves that excess Bcl-2 acts as a sink for upregulated Bim in resistant samples. Rather, the Bim binding partner data and ABT-737 Dex-sensitisation data combine to imply an important role for the relationship between Bim and Bcl-2 in GC activity within CLL cells.

Chapter 6: General Discussion

6.1 Discussion

Little was known about GC resistance in CLL prior to this investigation, and there are many unanswered questions in this field. In this study, molecular mechanisms that might contribute to GC resistance in CLL samples were explored by comparison of the most sensitive and resistant samples of a group of 46 primary CLL samples. In keeping with previous studies (Tiwari, Dong et al. 2005, Chandra, Gilbreath et al. 1997, McConkey, Chandra 1999), markers of apoptosis were elevated in CLL samples treated with Dex. These included PS exposure, PARP cleavage, and the previously reported activation of Bax and Bak (Bellosillo, Villamor et al. 2002). Signs of apoptosis were detectable as early as 4h following incubation with Dex, though killing was much more marked at 48h. Though caspase inhibition protected CLL samples from Dex-induced killing at 8h, at later timepoints this effect was not demonstrable. This confirms previous reports that caspases are not essential to cell death following initiation of apoptosis (Kroemer, Martin 2005). Consistent with the idea that GC resistance is not simply an extension of multi-drug resistance, fludarabine sensitivity did not appear to correlate with GC sensitivity. The observation that spontaneous apoptosis did not correlate with Dex sensitivity provided compelling evidence that GC sensitivity is not simply dependent upon constitutive survival signalling.

Intriguingly, samples from UM-*IGHV* patients were more prone to GC-induced killing than those from M-*IGHV* patients. Although similar findings were reported by others before and during this investigation (Aleskog, Tobin et al. 2004, Lindhagen, Norberg et al. 2009), this observation confirms that *IGHV* mutation status is relevant to GC resistance in CLL. The mechanisms by which *IGHV* mutational status might impact upon GC sensitivity are unclear and are not within the scope of this investigation. However, it has previously been suggested that UM-*IGHV* cases are more responsive to BCR stimulation than their M-*IGHV* counterparts, and it has been postulated that UM-*IGHV* cases may be chronically BCR activated (Zenz, Mertens et al. 2010). It is plausible that such cases are addicted to BCR-associated survival signals that can be disrupted by GC treatment. Thus, signalling pathways that are both activated by BCR stimulation and also implicated in GC signalling warrant further exploration in the setting of GC resistance. In support of this idea, whilst fludarabine sensitivity did not correlate with GC sensitivity, sensitivity to pro-survival factor inhibitors (MEK inhibitor and Bcl-2 inhibitor, Section 5.3) did appear to positively correlate with GC sensitivity, which suggests that GC-sensitive samples may have been more dependent upon pro-survival signalling than GC-resistant samples for their survival. As well as displaying higher levels of ZAP-70 and CD38, UM-*IGHV* samples have been shown to express higher levels of various genes relative to M-*IGHV* samples (Zenz, Mertens et al. 2010). These include the genes corresponding to targets of the kinase PKC, cell cycle regulators such as Cyclin-D1, and proteins involved in cellular metabolism (Rosenwald, Alizadeh et al. 2001). As described in Section 1.6.6, PKC, and various cell cycle regulating proteins have been implicated in GC sensitivity of certain haematopoietic cell

types, and so the activity of these proteins may contribute to the tendency for UM-*IGHV* samples to respond better to GC treatment than their M-*IGHV* counterparts.

Baseline expression of GRα and GRβ did not separate the two sensitivity groups, though *GRδ* mRNA level appeared to be higher in GC-sensitive samples. However, this difference could not be corroborated by protein levels due to the lack of an available antibody. This avenue warrants further exploration. Consistent with a previous study in CLL (Furman, Asgary et al. 2000), NF-κB was downregulated by Dex in sensitive samples. However, NF-κB was also downregulated in resistant samples. This provides novel insight and suggests that NF-κB is not a critical mediator of GC resistance in CLL cells. Consistent with these findings, pro-survival transcriptional targets of NF-κB were not preferentially downregulated in either sensitivity group, and an NF-κB inhibitor did not sensitise resistant samples to GC-induced killing. Pilot studies of Lck expression and GSK-3β expression and activation did not support a role for either kinase in GC sensitivity of CLL cells. Whilst Lck has previously been reported to be important in GC resistance in CLL cells (Harr, Caimi et al. 2010), the GSK-3β comparison was based on recent studies in ALL (Galliher-Beckley, Williams et al. 2008, Nuutinen, Ropponen et al. 2009). Furthermore, XIAP and c-IAP2 expression did not appear to segregate the two sensitivity groups, contrary to previous observations of the former in T cells (Conte, Liston et al. 2001).

Despite a clear difference in baseline ERK activation between the two sensitivity groups, MEK inhibitor did not sensitise resistant samples to GC-induced killing. Interestingly, Bim—a target of ERK, and apoptotic activator that is induced by

GCs in CLL (Iglesias-Serret, de Frias et al. 2007)—was induced in both groups of samples at the mRNA and protein levels. Bax and Bak baseline expression did not correlate with sensitivity to GC in this study, concordant with a previous study in CLL (Bosanquet, Sturm et al. 2002), but Bim and Bcl-2 did appear to be overexpressed in resistant samples. Mcl-1 levels were similar between the two groups with and without Dex treatment. However, Mcl-1 was not protected by a pan-caspase inhibitor despite observed protection from both apoptosis and PARP cleavage. This indicates that Mcl-1 is not essential for CLL cell survival at early timepoints and that Dex may downregulate Mcl-1 by a non-caspase dependent mechanism. This observation may be worth pursuing from a mechanistic perspective.

This study was the first to show the effect of Bim siRNA knockdown on GC-induced killing in CLL cells. As has been demonstrated in B-ALL cell lines (Abrams, Robertson et al. 2004), Bim inhibition protected sensitive samples from GCs. The predominant binding partner for Bim appeared to be Bcl-2, despite the fact that Bim can be bound/sequestered by various cellular proteins (Gomez-Bougie, Bataille et al. 2005). Thus, ABT-737—a mimetic of the BH3 domain that binds with high affinity to Bcl-2, Bcl-xl and Bcl-w (Certo, Del Gaizo Moore et al. 2006)—appeared to sensitise resistant samples to treatment with GC. Whilst synergy between these two agents has been suggested previously in CLL samples (Mason, Khaw et al. 2009), ABT-737 attenuation of Bcl-2 has not previously been linked to alleviation of GC resistance in CLL. A summary of the findings of this investigation is shown in Figure 6.1.

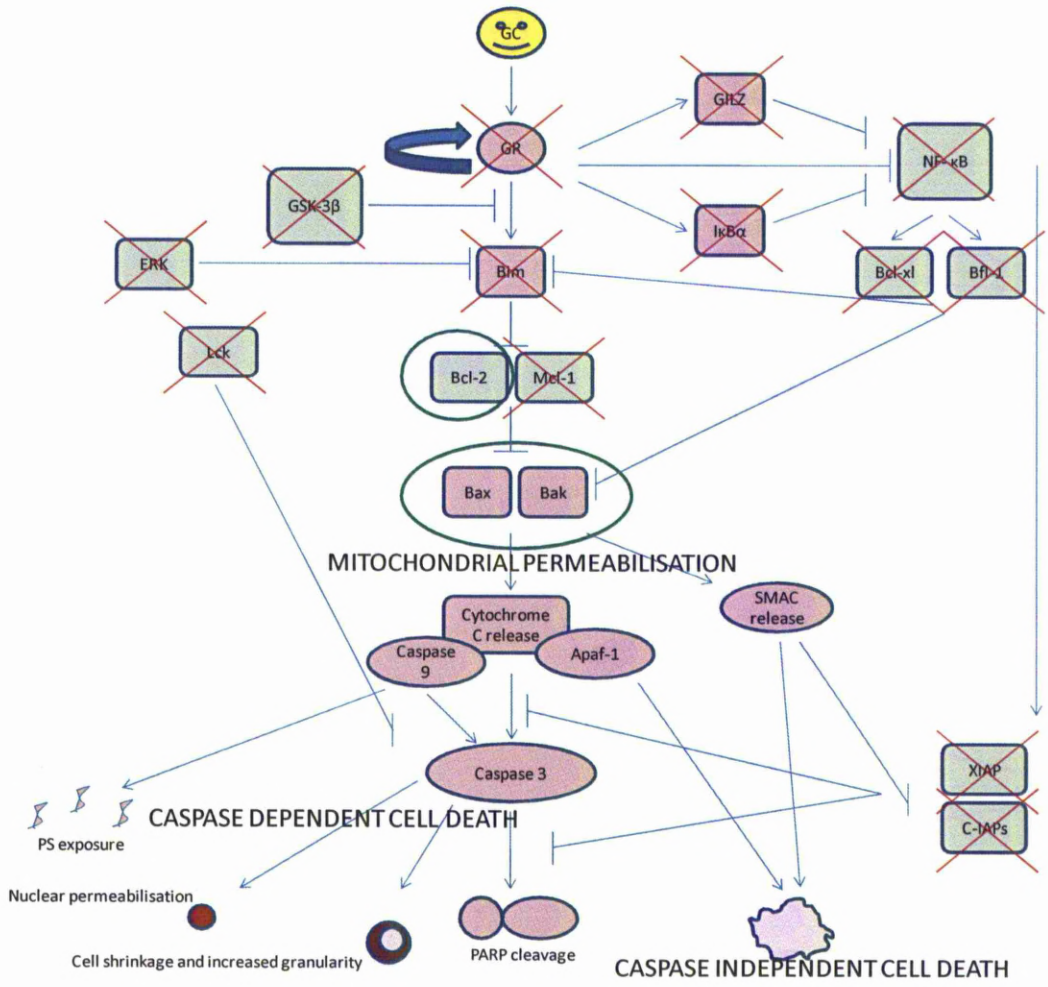


Figure 6.1. Model of GC-induced killing and resistance in CLL cells. Red entities predominantly possess proapoptotic function when activated, whilst green entities predominantly possess prosurvival function when activated. Red crosses highlight signalling entitities that do not appear to be imporant for GC resistance according to this investigation, whilst green ovals highlight points of interest. Clear differences between GC-sensitive and GC-resistant CLL samples were not observed with respect to GR expression and transactivation, NF-κB activity, kinase expression, or IAP expression. Sensitive samples did show a greater degree of apoptotic activation than resistant samples, as far upstream in the killing mechanism as Bax and Bak activation. Resistant samples showed higher expression of anti-apoptotic Bcl-2, but not Mcl-1, and Bcl-2 was found to coimmunoprecipitate with the GC-induced proapoptotic Bim. The Bcl-2 inhibitor ABT-737 increased GC-induced killing in resistant samples, and thus Bcl-2 sequestration of Bim is likely to contribute to GC resistance in CLL.

6.2 Strategic limitations

To define GC resistance in CLL patients refractory to GC treatment might be more informative than to screen samples for sensitivity *in vitro* and to then probe potential killing mechanisms. One of the reasons for this is that the *in vitro* test that this investigation was based on did not mimic the potential *in vivo* microenvironmental protection of CLL cells via localisation of cells within protective physiological compartments. Therefore, it might be more informative to purify CLL cells from the various lymphoid compartments of GC-resistant patients, for comparison to CLL cells from GC-responsive patients. This type of investigation was beyond the scope of this thesis, but is certainly worthy of consideration, in order to provide increased clinical relevance.

Regardless of whether samples from patients displaying GC resistance at the clinical level were used, without a clear mechanism of killing to probe, the detection of defects in resistant samples was likely to prove difficult in this study without a large cohort of samples to increase the power of statistical analyses. To screen the entire cohort at each stage of this investigation would certainly have been more informative. However, this would have been impractical, particularly with respect to the more time consuming assays.

Perhaps the most significant confounding factor in this study was the lack of correlation between GC sensitivity at 24 and 48h. This suggests that GC-induced killing is a dynamic process, and thus the experiments that use timepoints of 24h or less may not have provided a fair reflection of events at 48h. Perhaps a better

approach would to be contrast early events in sensitive samples with later events in resistant samples.

A broader approach to the question of whether GR-mediated alterations to the transcriptome contribute to GC resistance in CLL could be taken by conducting microarray experiments and to then validate interesting targets by further screening using multiple timepoints. However, such assays are complex, as is the analysis of the data produced, which may explain why no such analyses have been performed in CLL as yet. Furthermore, as Chapter 1 explains, there is increasing evidence that GCs are not entirely dependent upon changes in the transcriptome to induce cell death.

6.3 Future work

Along with the potential avenues of further investigation outlined in Sections 6.1 and 6.2, more pressing questions remain regarding the nature of Bcl-2 mediated GC resistance in CLL. Ideally, the experiments alluded to in Section 5.4 would be completed. To summarise, it would be helpful to:

- Increase *n* for Bcl-2 expression versus Dex response
- Show the effects of Bcl-2 and Bim overexpression on GC-induced killing
- Perform sub-cellular fractionation and confocal microscopy to localise Bim in CLL samples w/wo Dex treatment
- Identify the phosphorylation status of Bim in samples w/wo Dex treatment
- Perform reciprocal IPs for proteins not found to associate with Bim following Bim IP

- Show that Bax and Bak of GC-resistant samples can be activated by non-GC cytotoxic agents
- Perform formal tests for synergy with Dex and ABT-737

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